Molecular cloning of benzophenone synthase from *Hypericum calycinum* cell cultures and attempts toward transformation of *Hypericum perforatum*

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

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eingereicht am: 23.03.2011
mündliche Prüfung (Disputation) am: 08.06.2011

Druckjahr 2011

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge

Zodi R., Beuerle T., Beerhues L.: Benzophenone synthase from *Hypericum calycinum* cell cultures: cDNA cloning and functional expression (Poster). The 58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research. Berlin, Germany, 29th August- 2nd September 2010.

Zodi R., Beuerle T., Beerhues L.: Benzophenone synthase from *Hypericum calycinum* cell cultures: cDNA cloning and functional expression (Poster). Jahrestagung 2010 der Deutschen Pharmazeutischen Gesellschaft. Braunschweig, Germany, 4-7th October 2010.

Kwiecien I., Rogala I., **Zodi R.**, Beerhues L., Ekiert H.: Free phenolic acids in the biomass of *Hypericum perforatum* L. forms cultured *in vitro* - an HPLC analysis (Poster). The 7th International Symposium on Chromatography of Natural Products. Lublin, Poland, 14-17th June 2010.

Tocci N., Valletta A., **Zodi R.**, Liu B., Beerhues L., Pasqua G.: *Hypericum perforatum* subsp. *angustifolium in vitro* as a model for studying the biosynthetic pathway of xanthones (Poster). 105° Congresso S.B.I., Società Botanica. Mailand, Italy, 25-28th August 2010.

Abstract

Hypericum perforatum (St. John's wort; Clusiaceae) is a medicinal plant used for the treatment of mild to moderate depression. Four classes of secondary metabolites (hyperforins, hypericins, xanthones and flavonoids) are involved in the antidepressant activity and exhibit different pharmacological properties. Polyketide synthases (PKSs) type III catalyse the formation of these active constituents using different starters, different number of added extender and different intermolecular cyclization mechanism. The transformation of *H. perforatum* may examine the biosynthetic pathway of the different secondary metabolites, especially hyperforin, which is the main active constituent of *H. perforatum*. An example used in the transformation is *Agrobacterium*-mediated transformation. *A. tumefaciens* can transfer benzophenone synthase (BPS) gene from related species (*H. androsaemum*) into *H. perforatum*. This can interact with the hyperforin formation pathway, producing a hyperforin derivative, which may exhibit similar pharmacological activities but fewer side effects than hyperforin.

Benzophenone synthase catalyses the condensation of benzoyl-CoA with malonyl-CoA forming phlorbenzophenone, which is precursor of prenylated derivatives and xanthones. Xanthones are commonly minor constituents of *H. perforatum*. 1, 3, 6, 7-Tetrahydroxy-8-prenylxanthone was identified in yeast-treated cell cultures of related species; *H. calycinum*. A full-length cDNA encoding BPS with an ORF of 1200 bp was cloned from elicitor-treated cell cultures. The enzyme was functionally expressed and characterized. It shared 97.7% identity with BPS from *H. androsaemum.* It preferred benzoyl-CoA as starter substrate (k_m 10.83 µM). *p*- Coumaroyl and acetyl-CoAs were not accepted.

Zusammenfassung

Hypericum perforatum (Johanniskraut; Clusiaceae) ist eine Heilpflanze, die zur Behandlung von leichten bis mittelschweren Depressionen eingesetzt wird. Vier Klassen von sekundären Metaboliten (Hyperforine, Hypericine, Xanthone und Flavonoide) sind an der antidepressiven Wirkung beteiligt und weisen unterschiedliche pharmakologische Eigenschaften auf. Polyketidsynthasen (PKSs) vom Typ III katalysieren die Bildung dieser Wirkstoffe mit verschiedenen Startern, verschiedener Anzahl der Extender und verschiedenen intermolekularen Zyklisierungsmechanismen. Die Transformation von *H. perforatum* kann den Biosyntheseweg der verschiedenen sekundären Metabolite prüfen, insbesondere den des Hyperforins, das den wichtigsten aktiven Bestandteil von *H. perforatum* darstellt. Für die Transformation wurde u.a. die *Agrobacterium*-vermittelte Transformation angewendet. *A. tumefaciens* kann das Benzophenonsynthase (BPS)-Gen aus verwandten Arten (*H. androsaemum*) auf *H. perforatum* übertragen. Dies kann mit dem Biosyntheseweg von Hyperforin interagieren, wodurch ein Hyperforinderivat produziert werden kann, das ähnliche pharmakologische Aktivitäten, aber weniger Nebenwirkungen als Hyperforin zeigen könnte.
Die Benzophenonsynthase katalysiert die Kondensation von Benzoyl-CoA mit Malonyl-CoA und anschließend die Bildung von Phlorbenzophenon, was die Vorstufe von prenylierten Derivaten und Xanthonen ist. Xanthone sind Nebenbestandteile in *H. perforatum*. 1, 3, 6, 7-Tetrahydroxy-8-prenylxanthon wurde in mit Hefe behandelten Zellkulturen von verwandten Arten, *H. calycinum*, identifiziert. Eine cDNA in voller Länge, die für die BPS kodiert, wurde mit einem ORF von 1200 bp aus Elicitor-behandelten Zellkulturen kloniert. Das Enzym wurde funktionell exprimiert und charakterisiert. Es wies 97,7% Identität mit der BPS aus *H. androsaemum* auf. Es bevorzugte Benzoyl-CoA als Startersubstrat (k_m 10,83 µM). *p*-Coumaroyl-CoA und Acetyl-CoA wurden nicht akzeptiert.

ACKNOWLEDGMENT

First of all, I would like to express my deep and sincere gratitude to my supervisor Professor Dr. Ludger Beerhues for giving me the opportunity to work on this project, for his abundant help, invaluable assistance, immense knowledge, support and guidance throughout my work and living in Braunschweig.

I am thankful to Professor Dr. Ute Wittstock for her interest in this work and for taking over the co-referee.

Grateful thanks go to Dr. Benye Liu for his guidance, constructive discussion, advises and suggestions.

My appreciation is given to Dr. Rainer Lindigkeit for his help with software problems, for his great encouragement, support and advises during this work.

Special thanks are extended to Dr. Till Beuerle for his help with chemical analysis, for the useful discussions, kind support and friendly working conditions.

I want to say a big thank you to Dr. Mariam Gaid for her great help, excellent advices and patience during the preparation of this thesis.

My warm and deep thanks to all my colleagues and co-workers of the Institute of Pharmaceutical Biology, TU-Braunschweig. I especially thank Ines Rahaus for the DNA samples sequencing and the interesting discussions. Cornelia Hüttner, Noemi Tocci, Andreas Müller, Maren Lütge and Doris Glindemann for their assistance and the pleasant working atmosphere.

My sincere thanks are to my family for the encouragement and the continuous support during my life.

Finally, I would like to express my heartfelt thanks to my wife Nadine Zekert for her love, support and understanding.

Contents

List of abbreviations:

Amu	Atomic mass unit
APS	Ammonium persulphate
Au	Absorption Unit
BIS	Biphenyl synthase
BAP	6-Benzylaminopurine
bp	Base pair
BPS	Benzophenone synthase
BSA	Bovine serum albumin
BUS	Isobutyrophenone synthase
cDNA	Complementary deoxyribonucleic acid
CHS	Chalcone synthase
CoA	Coenzyme A
2,4-D	2,4-Dichlorophenoxyacetic acid
ddNTP	2´,3´-Dideoxynucleoside triphosphate (terminator nucleotide)
dH_2O	Distilled water
DEPC	diethylpyrocarbonate
DOC	Deoxycholic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	1,4-Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylendiamine-tetraacetic acid
EPI	Enhanced product ion scan
ESI-MS	Electrospray ionization- mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
GTC	Guanidinium thiocyanate
GUS	β -Glucuronidase enzyme
h	hour
6x His	Hexa-histidine tag
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid

IPTG	Isopropyl *β*-D-1-thiogalactopyranoside
KDa	Kilo Dalton
*K*m	Michaelis constant
Kb	Kilo base pair
LB	Luria Bertani
LS	Linsmaier and Skoog
MCS	Multiple cloning site
Min	Minute
M-MuLV	Moloney Murine Leukemia Virus
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
NAA	Naphthaleneacetic acid
NaAc	Sodium acetate
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PKSs	Polyketide synthases
RACE	Rapid Amplification of cDNA Ends
rpm	Revolution per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
Sec	Second
SMART	Switching mechanism at 5` end of RNA template
S.s.	Stock solution
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA buffer
TCA	Trichloroacetic acid
TEMED	*N,N,N',N'*- tetramethylethylene diamine

TLC	Thin layer chromatography
T_m	Melting temperature
Tris	Tris-(hydroxymethyl)aminomethan
V	Volt
X-Gal	5-Bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside
YEB	Yeast extract broth
ε	Extinction coefficient

Amino acids:

A=Ala	Alanine
C=Cys	Cysteine
D=Asp	Aspartic acid
E=Glu	Glutamic acid
F=Phe	Phenylalanine
G=Gly	Glycine
H=His	Histidine
I=Ile	Isoleucine
K=Lys	Lysine
L=Leu	Leucine
M=Met	Methionine
N=Asn	Asparagine
P=Pro	Proline
Q=Gln	Glutamine
R=Arg	Arginine
S=Ser	Serine
T=Thr	Threonine
V=Val	Valine
W=Trp	Tryptophan
Y=Tyr	Tyrosine

Nucleotides:

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

1. Introduction

1.1. *Hypericum perforatum* L.

H. perforatum (St. John's wort) (Fig. 1.1) belongs to the family Clusiaceae (formerly Hypericaceae). It is well known since ancient times as an important medicinal plant of the genus *Hypericum*, which includes about 450 species worldwide. The genus name *Hypericum* probably comes from the two Greek words, hyper = over and eikon = image. This expression is an indication that plants of this genus were believed to ward off evil or bad luck. The species name *perforatum* is related to the perforated appearance of the leaves when held in light due to the occurrence of translucent dots (Czygan, 1993 and 2003). The common name of the plant comes from its traditional flowering and harvesting around St. John's day, June 24. The plant reaches a height of 30 to 80 cm and blooms from June to August. The leaves contain translucent glands and red-black cell clusters accumulating hyperforin and hypericin, respectively (Fig. 1.2). The petals of the bright yellow-orange flowers are peppered with the black dots (Fig. 1.3). When these black dots are rubbed between the fingers, the skin becomes red because the oil contains the pigment hypericin. *H. perforatum* extract is widely known as herbal medicine for the treatment of depression.

Fig. 1.1. *Hypericum perforatum* L. (from Wikimedia Commons)

Fig. 1.2. *Hypericum perforatum* leaf (from Wikimedia Commons)

Fig. 1.3. *Hypericum perforatum* flowers (from Wikimedia Commons)

1.2. Constituents and pharmacological properties

H. perforatum is one of the best studied medicinal plants. Extracts from *H. perforatum* contain several groups of secondary metabolites with different pharmacological activities. The main classes of active constituents are shown in Table 1.1. *H. perforatum* contains xanthones and flavonoids in addition to hyperforins (Fig. 1.4) and hypericins (Fig. 1.5).

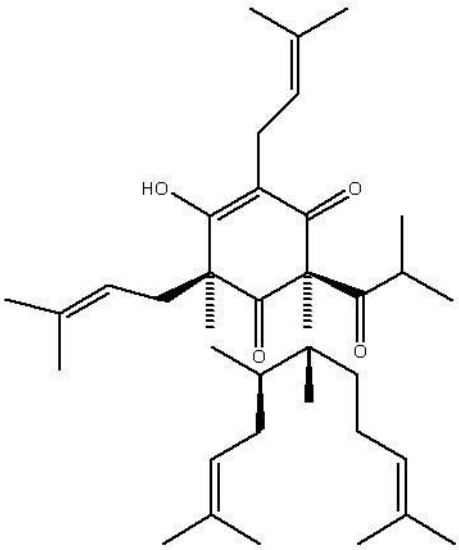


Fig. 1.4. Hyperforin

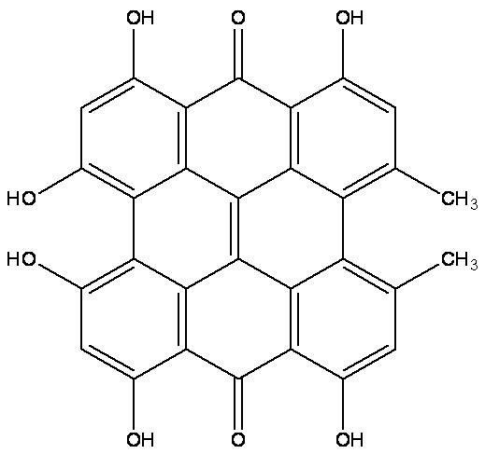


Fig. 1.5. Hypericin

Table 1.1. Main classes of active constituents of *Hypericum perforatum*

Chemical group	**Examples of compounds**
Flavonoids	Quercetin, rutin
Biflavones	Amentoflavon
Prenylated phloroglucinols	Hyperforin, adhyperforin
Naphthodianthrones	Hypericin, pseudohypericin
Xanthones	1,3,6,7-Tetrahydroxyxanthone

1.2.1. Flavonoids

Several flavonoid compounds (2-4% DW) such as flavonoid aglycones like quercetin (Fig. 1.6) and flavonoid glycosides like rutin (Fig. 1.7) are found in the upper parts of the plant. They give the flowers the attractive colour for attracting insects and protecting tissues from UV radiation (Winkel-Shirley, 2001). Beside their antioxidant activity they are discussed to play a role in the antidepressant activity of the total extract of *H. perforatum* (Butterweck, 2003).

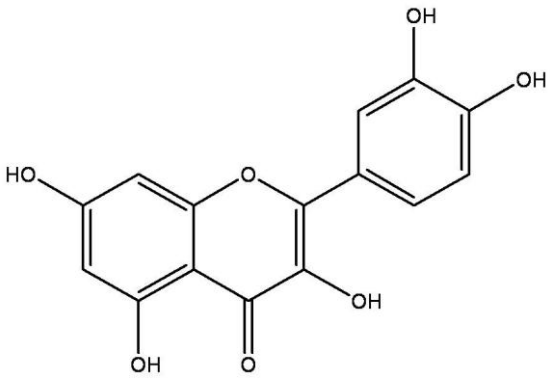


Fig. 1.6. Quercetin

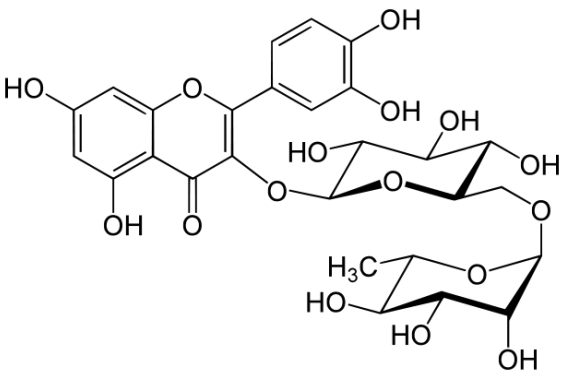
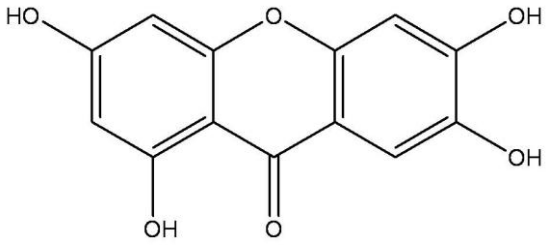


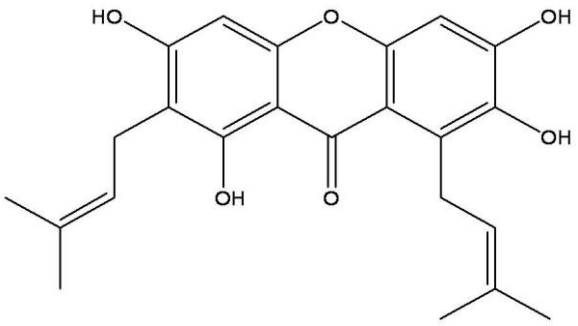
Fig. 1.7. Rutin

1.2.2. Xanthones

Xanthones such as 1,3,6,7-tetrahydroxyxanthone and γ-mangostin (Fig. 1.8) are found in small amounts in the upper parts of the plant. However, the roots contain higher amounts of xanthones (Greeson *et al*., 2001). Xanthones exhibit well known pharmacological properties. They have been described as strong scavengers of free radicals (Jiang *et al*., 2004). Other pharmacological properties of xanthones include anti-inflammatory (Banerjee *et al*., 2000) and anticancer (Ito *et al*., 2003) activities. In addition, xanthones exhibit antitumour, anti-HIV and antimicrobial activities (Beerhues *et al*., 2009).
Xanthones are induced in the plant in response to infection and provide a defense mechanism against stress (Abd El-Mawla *et al*., 2001; Franklin *et al*., 2009).



1,3,6,7-Tetrahydroxyxanthone



γ-Mangostin

Fig. 1.8. Xanthones from *Hypericum perforatum*

1.2.3. Naphthodianthrones

Hypericin (Fig. 1.5) and pseudohypericin are found in the red cell clusters of the leaves and the flowers (Piovan *et al*., 2004). They are responsible for the red colour of *H. perforatum* oil. For long time hypericin was thought to be the main active ingredients of *H. perforatum.* Hypericin exhibits antiviral and antitumor activities (Sirvent *et al*., 2003). However, it also causes cell photosensitization due to its chemical structure which is able to absorb UV radiation (Colasanti *et al*., 2000). Photosensitization may be a side effect during treatment with St. John's wort extract.

1.2.4. Hyperforin

Hyperforin (Fig. 1.4) is a bicyclic polyprenylated acylphloroglucinol derivative and is the main active constituent of *H. perforatum* (Beerhues, 2006). Hyperforin mainly exists in the ripe fruits and in the colourless glands of the leaves and the flowers. It exhibits an *in vitro* antitumoral activity (Schempp *et al*., 2002). The compound acts as antibacterial agent against gram-positive bacteria (Schempp *et al*., 1999). Due to its antiinflammatory activity *H. perforatum* extract is recommended for treatment of inflammatory skin disorders (Schempp *et al*., 2000). Hyperforin is also involved in the induction of cytochrome P450 enzymes such as CYP3A4. This monooxygenase is involved in the hepatic drug metabolism of more than 50% of all drugs, leading to drug-drug interactions (Moore *et al*., 2000). Clinically relevant interactions occur with drugs such as cyclosporine, digoxin, warfarin (Madabushi *et al*., 2006), immunosuppressants and HIV protease inhibitors (Beerhues, 2006).

The best studied pharmacological activity of hyperforin is the antidepressant property. Hyperforin inhibits the reuptake of neurotransmitters such as serotonin, dopamine, noradrenaline, glutamate and gamma-aminobutyric acid (GABA) (Müller, 2003) (Fig. 1.9). Hyperforin does not interact directly with the transmitter transporters but elevates the intracellular sodium concentration, thereby inhibiting the gradient-driven neurotransmitter reuptake (Singer *et al*., 1999). This mechanism explains the broad-band reuptake inhibitory activity. Synthetic antidepressants are competitive inhibitors of one or maximally two transporters at the transmitter binding sites (Beerhues, 2006).

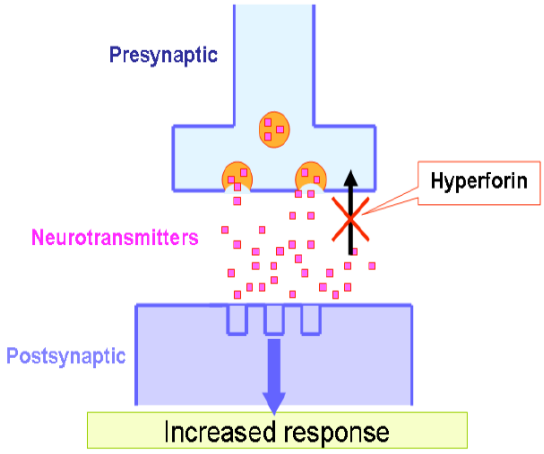


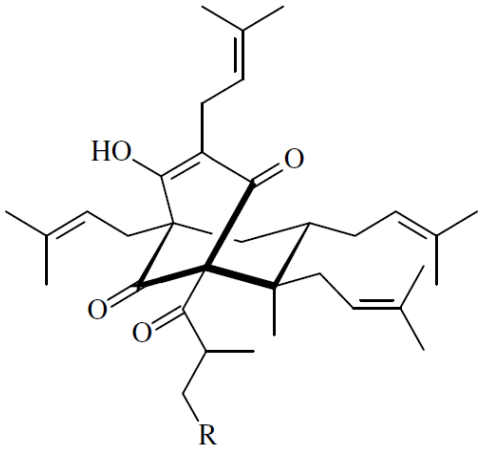
Fig. 1.9. Mode of the antidepressant action of *Hypericum perforatum* (from Capasso *et al.* 2003)

1.3. *Hypericum calycinum* L.

Another species of the genus *Hypericum is H. calycinum* (Fig. 1.10). This plant is an always green shrub. Alcoholic extracts from *H. calycinum* and *H. perforatum* exhibited similar effects on the CNS in mice (Öztürk, 1997).

Cell cultures of *H. calycinum* were established as an *in vitro* system for performing biochemical studies. They mainly contain adhyperforin and to a lower extent of hyperforin (Fig. 1.11), when grown in BDS medium in the dark (Klingauf *et al*., 2005). However, the concentrations of the hyperforins (approx. 0.03% DW) were markedly lower when compared to those in intact leaves (1.5% DW) (Sirvent *et al*., 2003).

Fig. 1.10. *Hypericum calycinum* (from Wikimedia Commons)



Hyperforin R = H
Adhyperforin R = CH_3

Fig. 1.11. Chemical structure of hyperforins

1.4. Polyketide synthases

Polyketide synthases (PKSs) are a class of enzymes that produce polyketides, a large group of secondary metabolites in bacteria, fungi and plants with wide pharmacological activities such as antibacterial, anticancer, immunosuppressant and antimycotic properties. The biosynthesis of the polyketides shows similarity with fatty acid biosynthesis involving malonyl-CoA.

Polyketide synthases are divided into three major groups: PKSs I, PKSs II and PKSs III. The best known member of the PKSs I group is erythromycin synthase, which consists of three multifunctional polypeptides (each about 350 kDa). Type II PKSs are likewise complexes that consist of several subunits and active sites. PKSs III are smaller than the two other PKSs (homodimers, about 84 kDa). They catalyze at a single active site per subunit all steps of the biosynthetic sequence: decarboxylation, condensation, cyclization and aromatization (Schröder, 1999). Three PKSs III that are involved in the biosynthesis of *Hypericum* secondary metabolites are shown in Fig. 1.12.

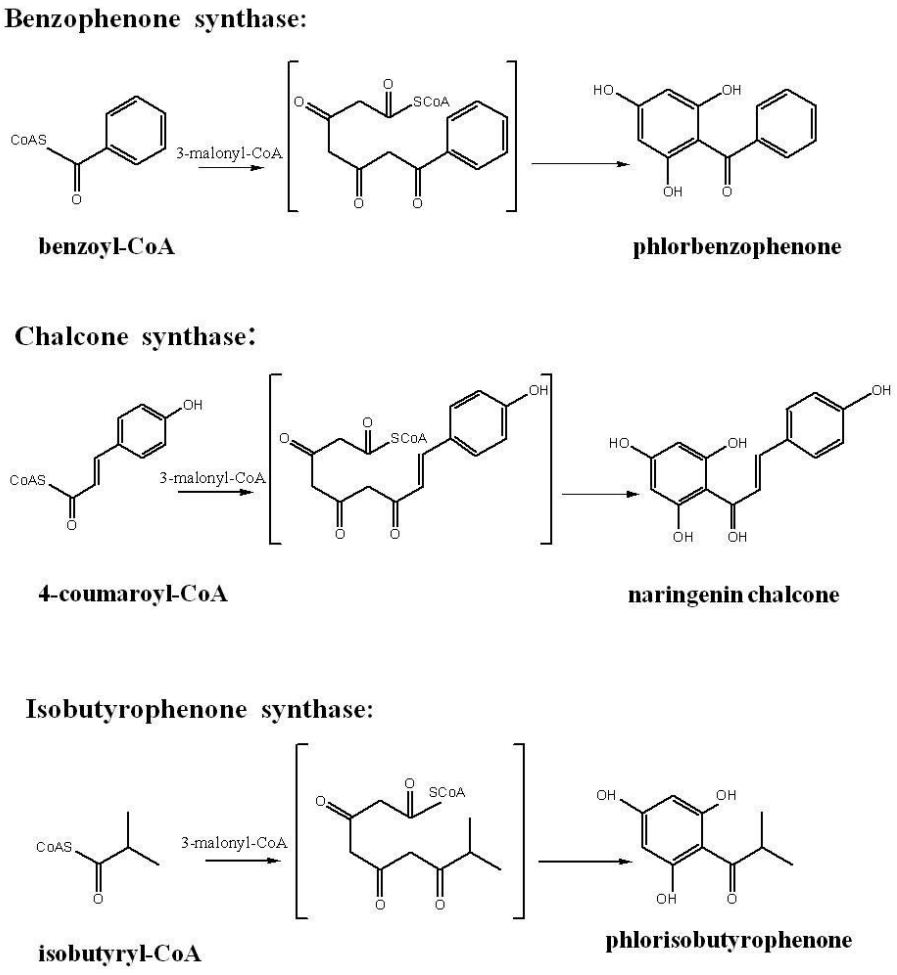


Fig. 1.12. Reactions of three type III polyketide synthases in *Hypericum* species (modified after Klingauf *et al*., 2005)

1.4.1. Benzophenone synthase

Benzophenone synthase (BPS) catalyzes the formation of phlorbenzophenone by condensation of benzoyl-CoA with three molecules of malonyl-CoA to give a tetraketide intermediate that cyclizes *via* intramolecular Claisen condensation into 2,4,6-trihydroxybenzophenone (Liu *et al*., 2003) (Fig. 1.12).

Phlorbenzophenone is the precursor of more complex polyprenylated benzophenone derivatives, such as garcinol and sampsonione A, which exhibit interesting pharmacological activities. Besides polyprenylation, phlobenzophenone derivatives can undergo intramolecular cyclization to give xanthones (Liu *et al*., 2003) (Fig. 1.13).

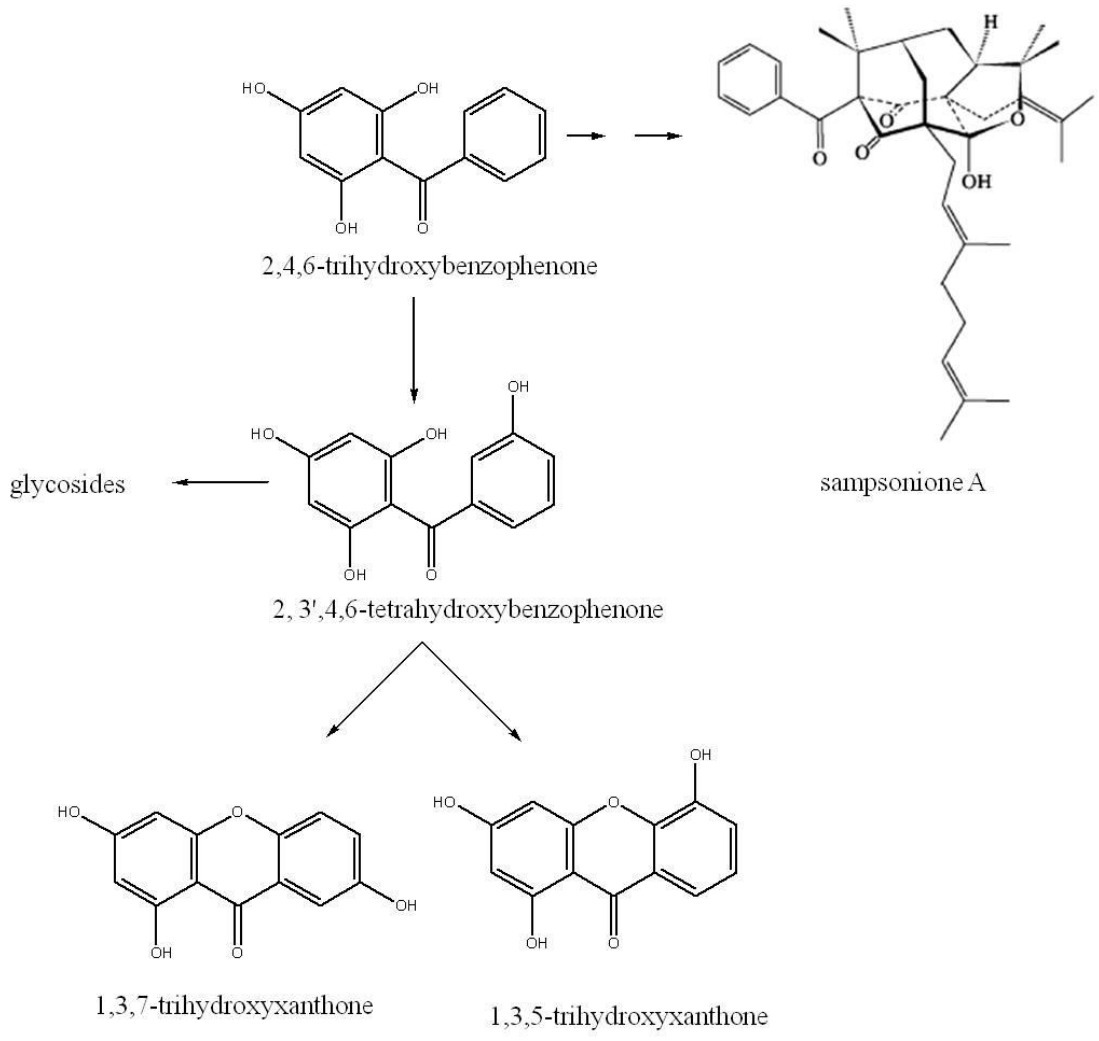


Fig. 1.13. Examples of pharmacologically active benzophenone derivatives and their biogenic relations (modified after Liu *et al*., 2003)

1.5. Plant genetic engineering

Genetic engineering techniques are applied in numerous fields including pharmacy, biotechnology, agriculture and medicine. Examples of compounds produced in this way are insulin and human growth factors. Foreign gene constructs can be stably introduced into plant genomes by various techniques, such as electroporation, biolistics and protoplast fusion (Walden and Schell, 1990). Recent advances in plant biotechnology provide a powerful tool for the construction of genetically improved transgenic plants (Krügel *et al*., 2002).

A. tumefaciens is nowadays a key of modern plant genetic engineering. It is a gram-negative soil bacterium which can infect wounded plants, especially dicotyledons. It can express a part of the tumor-inducing information (Ti-plasmid) known as transfer DNA (T-DNA) into the plant cells, where it integrates into the plant genome (Zaenen *et al*., 1974; Montoya *et al*., 1977). This T-DNA is located within right and left boarders and includes auxin and cytokinin genes responsible of producing crown gall disease in the plant host cells (Fig. 1.14).

Fig. 1.14. Crown gall induced by *Agrobacterium tumefaciens* (from Wikimedia Commons)

The T-DNA also includes genes for the synthesis of opines that are used by the *Agrobacterium* as an important source of nitrogen and carbon. In contrast, *Agrobacterium* produces no benefit to the plant. The Ti plasmid also contains virulence (vir) genes which recognize plant signals and work to inject the T-DNA into the plant (Tzfira *et al*., 2006; Tzfira and Citovsky, 2006).

For genetic engineering, *Agrobacterium* is a suitable vehicle for insertion of new genetic information into the plant cell genome using an appropriate mechanism to get a genetic alteration in the host cell. In contrast to other transformation methods, the foreign DNA fragment is stably integrated into the plant genome.

1.6. Objectives of this work

H. perforatum is an important medicinal plant. Hyperforin is its main active constituent, has antidepressant activity on the basis of a new mechanism of action and is not available chemically.

The objectives of this work were:

1- To establish an *Agrobacterium*-mediated transformation system for *H. perforatum.* As an example of a gene to be transferred, BPS was selected. Systemic expression of BPS in transgenic *H. perforatum* might result in modification of hyperforin biosynthesis (Fig. 1.15) and lead to production of new derivatives, which may retain the pharmacological activity but exhibit less side effects and drug-drug interaction. The transfer of the BPS ORF might interact with the hyperforin biosynthetic pathway by starting from phlorbenzophenone instead of phlorisobutyrophenone, thereby producing a new constituent (Fig. 1.16).

2- To clone, heterologously express and characterize BPS from *H. calycinum* cell cultures.

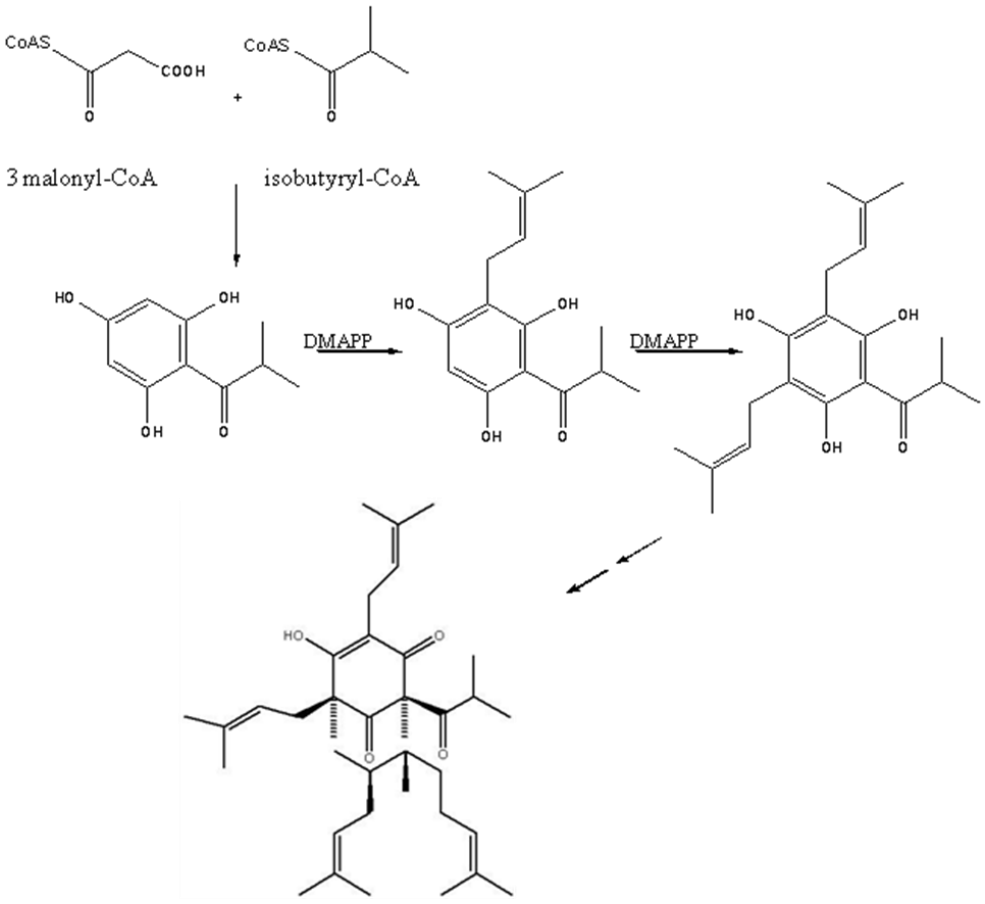
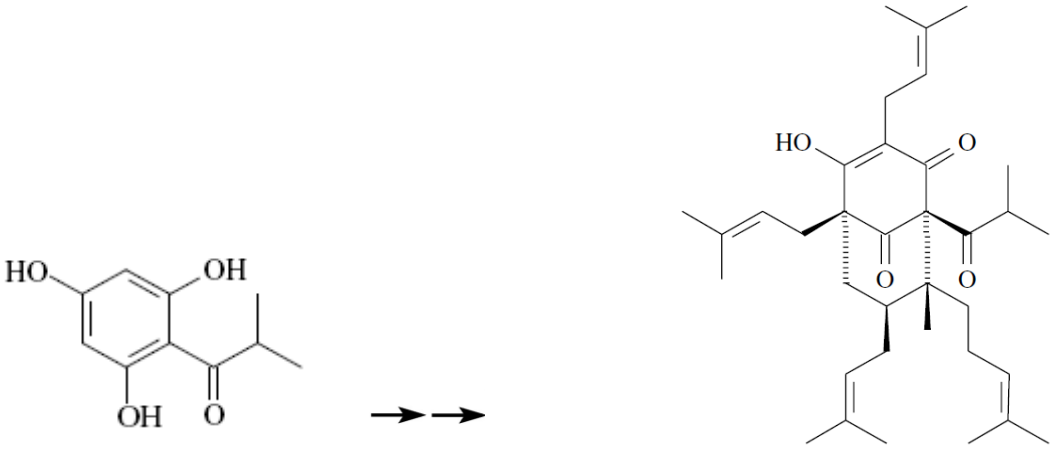
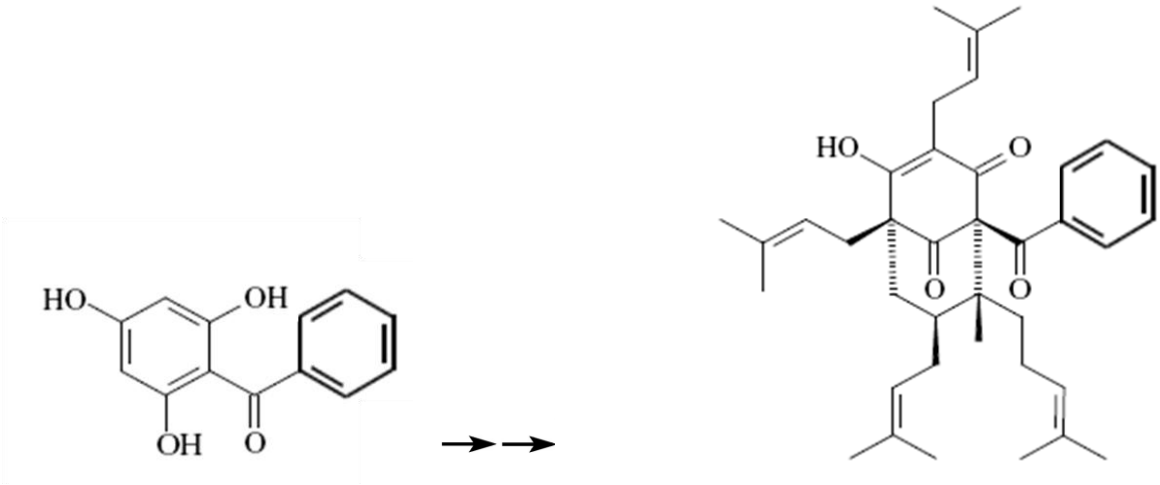


Fig. 1.15. Proposed hyperforin biosynthetic pathway (modified after Beerhues, 2006)



Phlorisobutyrophenone Hyperforin

A



Phlorbenzophenone Novel product

B

Fig. 1.16. A. Biosynthesis of hyperforin from phlorisobutyrophenone in wild type plants
B. Postulated formation of a derivative of phlorbenzophenone in transgenic lines

2. Materials

2.1. Plant material

2.1.1. *Hypericum perforatum in vitro* plant

Three seed varieties of *H. perforatum* (Helos, Topas and Elixir) were purchased from Richters Herbs, Canada. The seeds were surface-sterilized and germinated on solid Murashige Skoog (MS) medium (2.8.2). Seedlings were used in the transformation. Plants were grown at 25 °C on solid MS medium under the control of a 16 h light/8 h dark cycle and transferred every three to four months to fresh solid MS medium.

2.1.2. *Hypericum calycinum* cell suspension culture

Cell cultures of *H. calycinum* were grown as described previously for *H. androsaemum* (Abd El-Mawla *et al*., 2001). The undifferentiated cells of *H. calycinum* were grown in liquid LS medium (2.8.1) at 25 °C in the dark by constant shaking at 140 rpm; they were transferred every two weeks to fresh LS medium starting with 3 g cells in 50 ml LS medium.

2.2. Bacterial strains

Strain	Properties
E. coli DH5α	*F' φ80δlacZΔM15 end A1 hsdR17(rk⁻ mk⁺) supE44 thi-1 λ⁻ gyrA96 relA1 Δ(lacZYA-argFV169) deoR*
E. coli BL21 (DE3) pLysS	F⁻, *omp*T, *hsd*S (r_B^-, m_B^-), *gal*, *dcm*, (DE3), pLysS (Cam^R)
Agrobacterium tumefaciens C58C1	Binary vector pBIN19/BPS and pMP90 vector; resistant to kanamycin, rifampicin and gentamycin
Agrobacterium tumefaciens AGL1	Binary vector UGAB7; resistant to bialaphos
Agrobacterium rhizogenes LBA1334	Vector pRi 1855 including *rol*B gene; resistant to rifampicin

2.3. Vectors for transformation and expression

Plasmids for *Agrobacterium*	**Properties**
pBIN19/BPS	12 kb plasmid including *BPS* gene and kanamycin resistance gene
UGAB7	Includes *GUS* gene and bialaphos resistance gene
Plasmids for *E. coli*	**Properties**
pGEM-T easy	3 kb T-overhang vector used for the cloning of PCR products, includes the *lac*Z gene and an ampicillin resistance gene
pRSET B	2.9 kb vector used for protein expression including N-terminal His_6-tag and ampicillin resistance gene

2.4. Enzymes

DNA polymerases:	
Taq DNA polymerase, *Pfu* DNA polymerase	Fermentas
Endonucleases (restriction enzymes):	
*Eco*R1, *Nhe*I, *Kpn*I, *Hind*III	Fermentas
Enzyme for reverse transcription:	
RevertAid ™ H Minus M-MuLV RT	Fermentas
Other enzymes:	
T4-DNA Ligase, RNase A	Fermentas

2.5. Kits for molecular biology

DNeasy Plant Mini Kit	Qiagen
RNeasy Plant Mini Kit	Qiagen
DNA Purification Kit from gel	Analytic Jena
mRNA extraction kit	GE Healthcare
BigDye ® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems
DIG High Prime DNA Kit II for Northern Blot	Roche

2.6. Chemicals

Acetic acid	Roth
Acetone	Fisher Scientific
Acetosyringone (3,5-Dimethoxy-4-hydroxyacetophenone)	Fluka
Acrylamid	Gibco-BRL
Agar-Agar	Roth
Agarose	Roth
Ammoniumpersulfat	Bio-Rad
Ampicillin	Roth
BAP	Fluka
Benzoyl-CoA	Sigma
Chloramphenicol	Fluka
Coomassie-blue R 250 and G 250	Merck
D-Glucose	Fluka
DEPC	Sigma
DMSO	Fluka
dNTP	Fermentas
Dithiothreitol (DTT)	AppliChem
Ethyl acetate	Fisher Scientific
Formaldehyde 37% free from water	Merck
Glycerol	Roth
Glycine	Roth
IAA	Fluka
Imidazole	Roth
Isobutyryl-CoA	Sigma

IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Sigma
Kanamycin sulphate	Fluka
Maleic acid	Serva
Malonyl-CoA	Sigma
Methanol (HPLC)	Fisher Scientific
Methyl jasmonate	Serva
MOPS	Serva
NAA	Fluka
NaCl	Roth
Ni-NTA agarose	Qiagen
Peptone	Roth
Potassium dihydrogen phosphate	Roth
Ortho-phosphoric acid	Roth
Polyclar-AT	Serva
Seasand	Roth
Sodium acetate	Roth
Sodium dodecyl sulfate (SDS)	Roth
Sucrose	Fluka
TEMED	Bio-Rad
Trichloroacetic acid	Fluka
Tris	Roth
Yeast extract	Roth
X-Gal	Sigma

2.7. Equipments

Equipment	Type	Manufacturer
Autoclave	Vx-120	Systec GmbH Laborsystemtechnik
Balances	Large and small scale	Sartorius
Centrifuge	Universal 32R Biofuge 13 Sigma 1-15K	Hettich Heraeus Sepatech Sigma
Chemiluminescence Imager	HR 16 3200	Intas
Clean bench	LaminAir HLB 2472	Heraeus
Electrophoresis SDS	165-8000	BioRad
Electrophoresis DNA	power-pack 300	BioRad
HPLC	1525 Binary HPLC Pump Hewlett Packard HPLC system (1090 Series II) Dual absorbance detector 1200 Series	Waters Agilent Agilent
Hybridizer	HB-1000	UVP
Incubator-shaker	Minitron	Infors
pH Meter	Digital pH Meter 325	WTW
Spectrophotometer	ultraspec 1000	Pharmacia Biotech
Sequencer	ABI Prism	Appl.Biosystems
Speed vac	RVC 2-18	Christ
Thermo block	Dri-Block DB-3D	Techne
Thermo cycler	T-Proffessional Gradient	Biometra
Water bath	MW4	Julabo

2.8. Nutrient media

All media were autoclaved at 120 °C for 20 minutes before use.

2.8.1. Medium for cell suspension culture

Medium	Compositions		Notices
LS-liquid medium (Linsmaier and Skoog, 1965)	**I**		pH 5.5
	KNO_3	1900 mg/l	Store at RT
	NH_4NO_3	1650 mg/l	
	$CaCl_2 \cdot 2H_2O$	440 mg/l	
	$MgSO_4 \cdot 7H_2O$	370 mg/l	
	KH_2PO_4	170 mg/l	
	II		
	$MnSO_4 \cdot H_2O$	16.90 mg/l	
	$ZnSO_4 \cdot 7H_2O$	10.60 mg/l	
	KI	0.83 mg/l	
	H_3BO_3	6.20 mg/l	
	$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/l	
	$FeSO_4 \cdot 7H_2O$	27.80 mg/l	
	$CuSO_4 \cdot 5H_2O$	0.025 mg/l	
	$CoCl_2 \cdot 6H_2O$	0.025 mg/l	
	III		
	$Na_2EDTA.2H_2O$	41.30 mg/l	
	IV		
	Myo–Inositol	100 mg/l	
	Thiamine hydrochloride	0.4 mg/l	
	V		
	2,4-Dichlorophenoxyacetic acid	0.22 mg/l	
	1-Naphtylacetic acid	0.186 mg/l	
	VI		
	Sucrose	30 g/l	

2.8.2. Medium for *in vitro* plant cultivation

Medium	Compositions		Notices
MS-liquid medium (Murashige and Skoog, 1962)	**I**		pH 5.8
	NH_4NO_3	1650 mg/l	Store at RT
	KNO_3	1900 mg/l	
	$MgSO_4.2H_2O$	370 mg/l	
	$CaCl_2.2H_2O$	440 mg/l	
	KH_2PO_4	170 mg/l	
	II		
	H_3BO_3	6.2 mg/l	
	$MnSO_4.H_2O$	16.9 mg/l	
	$ZnSO_4.7H_2O$	8.6 mg/l	
	$FeSO_4.7H_2O$	27.8 mg/l	
	KJ	0.83 mg/l	
	Na_2MoO_4	0.25 mg/l	
	$CuSO_4.5H_2O$	0.025 mg/l	
	$CoCl_2.6H_2O$	0.025 mg/l	
	III		
	$Na_2EDTA.2H_2O$	3.2 mg/l	
	IV		
	Myo-Inositol	100 mg/l	
	Pyridoxine-HCl	0.5 mg/l	
	Thiamine-HCl	0.1 mg/l	
	Glycine	2 mg/l	
	Nicotinic acid	0.5 mg/l	
	V		
	Sucrose	20 g/l	
	VI		
For solid medium	Agar	10 g/l	
For the regeneration medium	BAP	0.5 mg/l	
	IAA	1 mg/l	

2.8.3. Media for bacterial culture

Medium	Compositions		Notices
LB medium	Bacto-pepton Yeast extract NaCl	10 g/l 5 g/l 10 g/l	pH 7 Store at RT
For solid medium	Agar	1.5%	

Medium	Compositions		Notices
SOC medium	Bacto-Peptone Yeast extract 1 M NaCl 1 M KCl Autoclave and add at 50 °C aliquots from the following sterile-filtered solutions: 2 M Glucose 2 M Mg^{2+} : ($MgCl_2.6H_2O$) 20.33 g + ($MgSO_4.7H_2O$) 24.65 g in 100 ml dH_2O	20 g/l 5 g/l 10 ml/l 2.5 ml/l 10 ml/l 10 ml/l	pH 7 Store at – 20 °C

Medium	Compositions		Notices
YEB medium	Bovine extract Yeast extract Peptone Sucrose $MgSO_4.7H_2O$	5 g/l 1 g/l 5 g/l 5 g/l 0.49 g/l	pH 7 Store at RT
For solid medium	Agar	1.5%	

2.9. Antibiotics stock solutions

Stock solutions of the following antibiotics were sterile-filtered (except for chloramphenicol) and stored at – 20 °C.

Antibiotic	Concentration
Ampicillin	100 mg/ml dH_2O
Chloramphenicol	35 mg/ml absolute ethanol
Cefotaxime	50 mg/ml dH_2O
Gentamycin	50 mg/ml dH_2O
Kanamycin	50 mg/ml dH_2O
Rifampicin	50 mg/ml DMSO
Streptomycin	50 mg/ml dH_2O

2.10. Phytohormones

Stock solutions of phytohormones (BAP, IAA, NAA and 2,4-D) were prepared by dissolving in absolute ethanol at 1 mg/ml and stored at 4 °C.

2.11. Elicitors

Elicitor	Stock solution (S.s.)	Application	Final concentration
Yeast extract	150 mg were dissolved in 1 ml dH_2O and sterile-filtered	1 ml of S.s. was added to 50 ml suspension culture	3 g/l
Methyl jasmonate	5 µl methyl jasmonate were dissolved in 440 µl absolute ethanol	100 µl of S.s. was added to 50 ml suspension culture	100 µM
Chitosan	1.25 mg were dissolved in 1 ml 1% (v/v) acetic acid, pH 5.5 adjusted with 5N NaOH, and sterilized by autoclaving	1 ml from S.s. was added to 50 ml suspension culture	25 mg/l

2.12. Oligonucleotides

All primers were synthesized in HPSF (high purity salt free) quality at MWG-Biotech AG (Ebersberg) Germany.

SMART II	5´-aag cag tgg taa caa cgc aga gta cgc ggg-3´
5'-CDS	5´-$(t)_{25}$ N_{-1}N-3'
3'-CDS	5´-aag cag tgg taa caa cgc aga gta $c(t)_{30}$ N_{-1}N-3´
T7	5´-gaa ttg taa tac gac tca cta tag-3´
SP6	5´-gat tta ggt gac act ata gaa tac-3´
pRSET B fwd	5´-gag acc aca acg gtt tcc ctc-3´
pRSET B rev	5´-cta gtt att gct cag cgg tgg-3´
*lac*Z fwd	5´ -agt cac gac gtt gta aaa cga cgg-3´
*lac*Z rev	5´ -tat gtt gtg tgg aat tgt gag cgg-3´
BPS Exp fwd	5´ -gca tgc tag cat ggc ccc agc aat gga ata-3´
BPS Exp rev	5´ -gca tgg tac ctc act gga gga tgg gga-3´
BPS *H.and* fwd	5´ -gga tca tat cat tca ccg ctc tt- 3´
BPS *H.and* rev	5´ -ata aca gaa cac tga aca caa tta tcc- 3´
Prob BPS fwd	5´ -acg ccc cca acg agt ccc acc- 3´
Prob BPS rev	5´ -ctc cat gac ggc ggc gat gtt atc- 3´
Prob CHS fwd	5´ -gtg ggc cca ccg aca ctc acc- 3´
Prob CHS rev	5´ -agt gag gct ctt ctc aac gtt ctt ag- 3´

2.13. Buffers and solutions

2.13.1. Buffer for protein extraction from cell culture

Kpi buffer + DTT	0.1 M KH_2PO_4 DTT 0.1 mM, should be freshly added pH 7

2.13.2. Buffer for enzyme assay

Kpi buffer	0.1 M KH_2PO_4 pH 7 Keep at RT

2.13.3. Solution to determine the protein amount

Bradford solution	Coomassie-Brilliant blue G250	100 mg
	Ethanol 96%	50 ml
	Phosphoric acid 85%	100 ml
	dH_2O	ad 1000 ml
	Dissolve Coomassie in ethanol, add phosphoric acid and complete the volume to 1 liter, filter the solution through filter paper and keep at 4 °C	

2.13.4. Solutions for protein precipitation

DOC solution	Dissolve 0.1% (w/v) sodium deoxycholic acid and 0.02% sodium azide in dH_2O
TCA solution	Dissolve 55% (w/v) trichloroacetic acid in dH_2O

2.13.5. Solution for PD_{10} column regeneration

NaOH 0.16 M	3.2 g NaOH dissolved in 500 ml dH_2O. Wash the PD_{10} column with 25 ml washing solution followed by water till the pH be neutral

2.13.6. Solution for transformation and selection of *E.coli*

IPTG 0.5 M	Dissolve freshly 6 mg IPTG in 500 µl dH_2O, sterile-filtrate and store at – 20 °C; the final concentration for the induction is 1 mM
X-Gal	5-Bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside 40 mg N,N`-Dimethylformamide 1 ml Use 40 µl per petridish and store at – 20 °C

2.13.7. Buffers for protein affinity purification

Lysis buffer pH 8	NaH_2PO_4 NaCl 20 mM Imidazole dH_2O	3.4 g 0.9 g 0.7 g ad 500 ml
Washing buffer pH 8	NaH_2PO_4 NaCl 50 mM Imidazole dH_2O	3.4 g 0.04 g 1.7 g ad 500 ml
Elution buffer pH 8	NaH_2PO_4 NaCl 250 mM Imidazole dH_2O	3.4 g 8.8 g 8.5 g ad 500 ml

2.13.8. Buffers for DNA isolation

Buffer 1	Tris-HCl	50 mM
	EDTA	10 mM
	RNase A	100 µg/ml
	pH 8	RNase A freshly added
Buffer 2	NaOH	0.2 M
	SDS	1% (w/v)
Buffer 3	Potassium acetate	2.55 M
	Adjust pH to 5.5 with glacial acetic acid	

2.13.9. Buffers for DNA gel electrophoresis

50x TAE buffer	Tris-HCl	2 M
	EDTA	0.05 M
	pH 8; prepare 1x TAE buffer for the DNA electrophoresis	
TBE buffer for sequencing gel	Tris-HCl	90 mM
	Boric acid	90 mM
	EDTA	2 mM
Loading buffer for sequencing gel	25 mM EDTA	1 ml
	Formamide	5 ml
	Dextran blue	0.1 mg
Sequencing gel	Urea	9 g
	10x TBE buffer	3 ml
	dH_2O	11.5 ml
	30% Acrylamide	3.75 ml
	TEMED	0.01 ml
	10% (w/v) APS	0.23 ml
DNA loading buffer	Xylencyanol	0.25 g
	Bromophenol blue	0.25 g
	Ficoll 400	25.00 g
	EDTA	1.46 g
	dH_2O	ad 100 ml

2.13.10. Buffers for protein gel electrophoresis (SDS PAGE)

Stacking gel (5%)	dH_2O	3.4 ml
	0.5 M Tris-HCl (pH 6.8)	0.63 ml
	Acrylamide/Bis 30%	0.83 ml
	10% (w/v) SDS	0.05 ml
	10% (w/v) APS	0.05 ml
	TEMED	5 µl
Separating gel (12%)	dH_2O	3.3 ml
	1.5 M Tris-HCl (pH 8.8)	2.5 ml
	Acrylamide/Bis 30%	4 ml
	10% (w/v) SDS	0.01 ml
	10% (w/v) APS	0.01 ml
	TEMED	4 µl
Protein loading buffer	dH_2O	2.7 ml
	0.5 M Tris-HCl (pH 6.8)	1 ml
	Glycerin	2 ml
	10% (w/v) SDS	3.3 ml
	β-mercaptoethanol	0.5 ml
	0.5% (w/v) Bromophenol blue	0.5 ml
10x Running buffer	Tris-HCl	15 g
	Glycin	72 g
	Na-SDS	5 g
	dH_2O	ad 500 ml
Coomassie-blue stock solution	Coomassie-blue R 250	0.5 g
	dH_2O	ad 50 ml
Staining solution	Coomassie-blue R 250 S.s.	25 ml
	Methanol	100 ml
	Acetic acid	20 ml
	dH_2O	ad 200 ml
Destaining solution	Methanol	30 ml
	Acetic acid	20 ml
	dH_2O	200 ml

2.13.11. Buffers for RNA gel electrophoresis

Gel running buffer	10x MOPS buffer Formaldehyde 37% RNase-free water	100 ml 20 ml 880 ml
10x MOPS buffer	MOPS NaAc EDTA RNase-free water pH 7	8.37 g 0.82 g 1.189 g ad 200 ml
5x RNA loading buffer	Saturated bromophenol blue solution 500 mM EDTA, pH 8.0 Formaldehyde 37% Glycerol 100% Formamide 10 x MOPS buffer RNase-free water	16 µl 80 µl 720 µl 2 ml 3084 µl 4 ml ad 10 ml

2.13.12. Buffers for Northern blot

Washing buffer	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20 °C); 0.3% (v/v) Tween 20
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5

3. Methods

3.1. Cultivation of *Hypericum perforatum* seeds

Seeds of three varieties of *H. perforatum* (Helos, Topas and Elixir) were surface-sterilized under the clean bench by incubation for not more than two minutes in 70% ethanol and then washed with autoclaved water. Seeds were finally sterilized with 6% sodium hypochlorite for five minutes and then washed with sterile water for several times. Seeds were dried on sterile filter paper and cultivated at 25 °C on solid hormone-free Murashige Skoog medium (MS) (2.8.2) under the control of a 16 h light/8 h dark period to initiate the starting material required for the transformation. Seeds started to germinate within three weeks and complete plant developed after 6 to 7 weeks. Plants were subcultivated every 7-8 weeks into a fresh solid MS medium.

3.2. Transformation of *Hypericum perforatum*

3.2.1. Preparation of *Agrobacterium tumefaciens* C58C1

Under sterile conditions *A. tumefaciens* C58C1 was picked from frozen culture using a sterile loop. Bacteria was spread on solid YEB plates containing kanamycin, gentamycin and rifampicin at final concentrations of 50 µg/ml for kanamycin and rifampicin and 20 µg/ml for gentamycin. Plates were incubated in dark at 28 °C overnight.
The next day one colony was picked up and transferred to 10 ml liquid YEB medium containing the same antibiotic composition. The *Agrobacterium* was grown by shaking in the dark at 25 °C and 130 rpm. At OD_{600} = 0.6-0.8, bacterial cultures were centrifuged at 5000 rpm for 10 minutes and the pellets were resuspended and incubated for 2 hours in 5 ml liquid MS medium pH 5.8 (2.8.2) containing 100 µM acetosyringone, which is one of the phenolic compounds to activate the vir genes on the Ti-DNA of the binary vector.

Various incubation times (1, 2, 12 and 24) h of *Agrobacterium* with different concentrations of acetosyringone (50, 100 and 200 µM) were applied to achieve an optimal condition for the transformation.

3.2.2. Infection of *Hypericum perforatum* with *Agrobacterium tumefaciens* C58C1

Different plant organs (leaves, stems and roots) were used as explants for the transformation. Leaves for example were separated from the stem and injured from different sites to get a wide contact surface with the *Agrobacterium* during the co-cultivation time, dipped into the activated bacterial solution for a short time, dried on sterile filter paper, transferred to antibiotic-free regeneration medium (MS solid containing 0.5 mg/l BAP and 1 mg/l IAA) (2.8.2) and incubated for two days at 25 °C in the dark. Cefotaxime (250 mg/l) was added after one week to the medium to get rid of the overgrowth of the bacteria. Kanamycin (50 mg/l) was added to perform selection of the explants; they were transferred every three weeks to a fresh regeneration medium including the same composition under the control of a 16 h light/8 h dark period.
Different co-cultivation times (1, 2, 12, 24 and 48 h) with the activated *Agrobacterium* (3.2.1) were applied to achieve optimal conditions for the transformation.

3.2.3. Infection with *Agrobacterium tumefaciens* AGL1

The *A. tumefaciens* hyper-virulence strain AGL1 was prepared for the infection in the same way as the *A. tumefaciens* strain C58C1 (3.2.1) except for the antibiotic composition (gentamycin 20 µg/ml, streptomycin 50 µg/ml and ampicillin 100 µg/ml). Different concentrations of acetosyringone were applied for different periods with leaves. They were transferred to the regeneration medium (2.8.2) and incubated for two days at 25 °C in the dark. Cefotaxime (250 mg/l) was added after one week to the medium to get rid of the overgrowth of the bacteria. The selection was done later with bialaphos at 5 mg/l. Infected explants were transferred every three weeks to a fresh regeneration medium under the control of a 16 h light/8 h dark period.

3.2.4. Infection with *Agrobacterium rhizogenes*

The *A. rhizogenes* strain LBA1334 is not disarmed as the *A. tumefaciens* strains. It can induce hairy roots in the infected cells by transferring the T-DNA to the host cells. An *A. rhizogenes* – 80 °C stock was spread on solid YEB medium (2.8.3) containing 50 µg/ml rifampicin and incubated at 28 °C in the dark. On the next day one colony was picked up, transferred to 10 ml liquid YEB medium including 50 µg/ml rifampicin and left to grow till OD_{600} = 0.6-0.8. The bacterial culture was centrifuged at 5000 rpm for 10 minutes. The bacterial pellet was resuspended and incubated for 2 hours in 5 ml liquid MS medium pH 5.8 containing 100 µM acetosyringone. Leaves of *H. perforatum* were cut and co-cultivated for different periods in the activated bacterial solution. Explants were dried on sterile filter paper and transferred to solid MS medium without phytohormones for one week at 25 °C in the dark. Cefotaxime (250 mg/l) was added to MS medium to inhibit the growth of *Agrobacterium*. The leaves were transferred every three weeks to hormone-free MS medium and kept in dark.

3.2.5. Vacuum infiltration for transformation

This technique was successfully used with several plants by decreasing pressure and allowing the bacterial solution to go inside the intercellular spaces. The *Agrobacterium* culture was grown as described previously (3.2.1), about 100 ml was prepared till OD_{600} = 0.8-1 and transferred to a sterile vacuum flask connected to a vacuum pump. Leaves of *H. perforatum* were separated from stems and added to the medium and submerged. Vacuum of 400 mm Hg was applied for about 15 minutes and air bubbles appeared during this time on the surface of the leaves. The pressure was then released immediately and the leaves were dried on sterile filter paper and incubated on the antibiotic-free regeneration medium for two days in the dark. They were sub-cultured every three weeks to fresh regeneration medium (2.8.2) containing cefotaxime 250 mg/l and kanamycin 50 mg/l.

3.3. Isolation of genomic DNA from *Agrobacterium tumefaciens*

One scoopful of the – 80 °C *A. tumefaciens* C58C1 storage was transferred into 5 ml liquid YEB (2.8.3) medium containing the appropriate antibiotics. The bacterial culture was incubated overnight by shaking at 140 rpm at 37 °C. The next day 2 ml of the new culture were harvested by centrifugation for 5 minutes at 5000 rpm. The bacterial pellet was then resuspended in 300 µl buffer 1 (2.13.8). Then 300 µl buffer 2 (2.13.8) were added to the bacterial suspension to lyse the cells and incubated on ice for not more than five minutes, followed by 300 µl buffer 3 (2.13.8). Samples were mixed gently and incubated on ice for 20 minutes. The solution was centrifuged for 15 minutes at 13000 rpm. The clear supernatant was transferred to a new reaction tube and extracted with an equal volume of chloroform. The DNA was precipitated from the aqueous phase by adding a 2/3 volume fraction of 2-propanol and centrifuged at 13000 rpm for 30 minutes. The pellet was washed with 500 µl 70% ethanol and the purified DNA was dried and redissolved in 50 µl dH_2O. This DNA includes genomic DNA, Ti plasmid and the binary vector pBIN19/BPS.

3.4. Isolation of plant genomic DNA

The isolation of genomic DNA was done by using the extraction and purification kit DNeasy plant mini kit (Qiagen), as described in the manufacturer's guidelines. One hundred milligram plant material were ground with liquid nitrogen to destroy the cell wall and allow the DNA to be resuspended after adding suitable buffer. RNase was added to digest RNA following lysis. Cell debris and precipitates were removed by centrifugation through a QIAshreder TM column. Binding buffer and ethanol were added to the clear lysate to allow DNA adsorption on the membrane in presence of high concentration of chaotropic salts, which removed water from hydrated molecules in solution, whereas contaminants such as proteins and polysaccharides were efficiently removed by two wash steps. Pure DNA was eluted with autoclaved water and stored at – 20 °C for further usage.

3.5. Determination of DNA and RNA concentrations

The concentration of nucleic acids was determined by measuring the absorbance value of the DNA or RNA samples at wavelength 260 nm. One unit absorbance value at wavelength 260 nm (ε_{260}) corresponds to 40 ng/µl single stranded RNA or 50 ng/µl double stranded DNA (Sambrook *et al*., 2001). A UV/Vis spectrophotometer was used to determine the concentration of DNA or RNA samples in a volume of 0.5 ml by using quartz cuvettes. The calculation was as follows: concentration (DNA or RNA) = ε_{260} x abs. x dilution factor. Proteins have absorbance at wavelength 280 nm and the ratio A_{260}/A_{280} is a purity sensor, which should be not less than 1.8. Contaminations with proteins or phenolics would reduce this value due to their absorbance at 280 nm.

3.6. Design of gene-specific primers

The primers generally should be able to recognize specifically a part of the cDNA sequence to allow the DNA polymerase to build a complementary sequence for this single strand DNA. The primers in this work were synthesized at MWG-Biotech, Germany. They should consist of 18-25 nucleotides and have a GC content of approximately 40-60% and a melting temperature 55-65 °C. When a restriction site is required to be added to primers to generate sticky ends, for example in case of over-expression using pRSET B vector, 4-10 nucleotides (depending on the restriction enzyme) should be added to the 5´ end of the primer to allow an efficient digestion of the ends of the cDNA fragment.

3.7. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method of nucleic acid synthesis, by which a particular segment of DNA can be specifically replicated by means of thermo-stable DNA polymerase (Saiki *et al*., 1988). At the first step, the double strands of DNA are denatured at 94 °C. The next step is the annealing of primers to their complementary sequence of the DNA template at a temperature which depends on the size of the primer and the number of its nucleotides. The third step is the elongation of the annealed primers by a DNA polymerase at 72 °C. This cycle will be repeated for many times to duplicate the desired DNA.

Generally the annealing temperature is 5 °C below the melting temperature (T_m) of the used primers. The melting temperature of primer can be adjusted as follows:

T_m = 4 °C x (number of G and C in the primer) + 2 °C x (number of A and T in the primer)

Standard PCR components are as follows**:**

DNA	1 µg
Forward primer (10 pmol/ul)	1 µl
Reverse primer (10 pmol/ul)	1 µl
10x reaction buffer with 20 mM $MgCl_2$	2.5 µl
dNTPs (10 mM)	1 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
dH_2O	ad 25 µl

The Standard PCR program was**:**

Step	Temperature °C	Time sec	Purpose
1	94	180	Denaturation
2	70	Pause	Hot start
3	94	45	Denaturation
4	T_m – 5 °C	60	Annealing
5	72	120	Elongation
6	72	600	Final Elongation
7	12	Pause	Stop

The steps 3 to 5 should be repeated 30 times before starting step 6.

In case of using *Pfu* DNA polymerase**:**

Buffer with $Mg(SO_4)_2$ was used instead of the one with $MgCl_2$; elongation temperature was decreased to 68 °C in the initial and the final elongations; elongation time was increased to 240 sec in initial and 1200 sec in final elongation.

3.8. DNA agarose gel electrophoresis

This method is used to separate DNA fragments due to their size during migration towards the anode by applying an electric field to move the negatively charged molecules through an agarose matrix. The gel was prepared in concentrations between 0.8-2% of agarose by boiling for short time in a microwave to dissolve the agarose in 1x TAE buffer (2.13.9), then cooling down to 60 °C. Ethidium bromide was added in a final concentration of 0.5 µg/ml and the solution was poured into the gel tray. All the steps involving the work with ethidium bromide should be done under the fume hood, because it acts as mutagen. Ethidium bromide binds to DNA and has fluorescence under UV. The samples were mixed with DNA loading buffer (2.13.9) and the gel was run for about 30 min in a gel chamber including 1x TAE buffer at a voltage of 120 V. A DNA marker was run in parallel to compare the size of the separated DNA fragments. The DNA bands were visualized in the gel by using an imager under UV light at 302 nm.

3.9. Purification of DNA from agarose gel

DNA fragment separated by electrophoresis were isolated under UV light for further applications such as restriction or ligation. PCR product purification from the gel was performed by using a gel extraction kit (Analytic Jena) according to the manufacturer's instructions .The piece of gel was dissolved in a 3-fold volume of buffer at 50 °C, bound to a column including a silica gel membrane and washed. The DNA was eluted thereafter with water from the column.

3.10. Elicitation of *Hypericum calycinum* cell cultures

To study the response of the cell culture to different elicitors (yeast extract, chitosan and methyl jasmonate), four-day-old cell cultures after sub-division were treated with these elicitors (2.11) separately. The cells were harvested every three hours after the onset of elicitation and either used directly to extract proteins, active constituents and RNA or stored for further usage at – 20 °C or – 80 °C.

3.11. Extraction of xanthones from *Hypericum calycinum* cell culture

After challenge of cells with different elicitors, it was noticed that cells responded only to yeast extract by forming xanthones. To isolate the xanthones and to study their accumulation after induction, 3 g cells were collected by filtration every three hours after the addition of yeast extract. They were homogenized for 10 minutes in a mortar by using 10 ml acetone and a low amount of sea sand. After filtration the residue was extracted again with 10 ml acetone for five minutes and filtrated again. Acetone was evaporated to dryness by using a rotary evaporator at 40 °C and 556 mbar. The residue was redissolved in l ml methanol for HPLC and filtered through a small filter paper. The solution was either stored at – 20 °C or an aliquot was injected directly into the HPLC.

3.12. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was applied to detect the formation of new products after treatment of *H. calycinum* cell cultures with different elicitors. TLC separation was applied by using aluminum sheets coated with silica gel (silica gel 60 F254 plates, 20 x 20 cm, layer thickness 0.2 mm; Merck, Darmstadt, Germany). The samples and the control were applied to the plate with a glass capillary and immersed in a TLC chamber containing the mobile phase. The detection was done under UV light.
The mobile phase composition was as follows:

Dichloromethane 89%	Methanol 10%	Formic acid 1%

3.13. High performance liquid chromatography (HPLC)

For HPLC separation of constituents from cell culture extracts and enzymatic products, a gradient elution was used. The mobile phase was a mixture of ethanol (HPLC grade) and dH_2O containing 0.1% phosphoric acid. The composition of the elution changed within the specified time intervals. The flow rate was 0.5 ml/min using the C18 column Hyperclone 5 µm (150 mm x 3.20 mm) (Phenomenex).

The HPLC gradient for the BPS and CHS products was as follows:

	Time (minute)	Methanol %	Water %	Wavelength (nm)
BPS	0	20	80	306
	3	20	80	
	17	50	50	
	23	100	0	
	25	100	0	
	28	20	80	
	34	20	80	

	Time (minute)	Methanol %	Water %	Wavelength (nm)
CHS	0	20	80	286
	3	20	80	
	17	50	50	
	23	100	0	
	25	100	0	
	28	20	80	
	34	20	80	

The HPLC gradient for the active constituents from cell cultures was as follows:

	Time (minute)	Methanol %	Water %	Wavelength (nm)
Xanthones	0	30	70	254
	3	30	70	
	30	70	30	
	33	70	30	
	36	100	0	
	39	100	0	
	41	30	70	
	45	30	70	

	Time (minute)	Methanol %	Water %	Wavelength (nm)
Flavonoids	0	30	70	290
	3	30	70	
	30	70	30	
	33	70	30	
	36	100	0	
	39	100	0	
	41	30	70	
	45	30	70	

3.14. HPLC-MS analysis

Cell-free extract from treated *H. calycinum* cells was analyzed using HPLC-ESI-MS.

Instrumental:

Mass spectrometer: Applied Biosystems 3200 Q TRAP, Turbo V Ion Source

Software: Analyst 1.4.2

Collision gas: N2

HPLC: Agilent Series 1200

Column: Nucleosil C_{18} 100-5 (25 x 0.4 cm)

HPLC-Gradient:

Time (minute)	Methanol %	Water %	Wavelength (nm)
0	30	70	
3	30	70	
30	70	30	254
33	70	30	
36	100	0	
39	100	0	
41	30	70	
45	30	70	

MS-Parameter/Experiment	**Q1+**	**EPI+**
Curtain gas	10 ml/min	10 ml/min
Collision gas	-	High
Ion spray voltage	5500 V	5500 V
Temperature	350	350
Ion source gas 1	51 ml/min	51 ml/min
Ion source gas 2	50 ml/min	50 ml/min
Declustering potential	25 V	25 V
Entrance potential	3 V	3 V
Collision energy	-	49 V
Collision cell exit potential	-	4 V

3.15. GC-MS analysis

Gas chromatography is a powerful and sensitive separation tool for volatile compounds. The stationary phase was a ZB5MS column (Phenomenex, Aschaffenburg, Germany) and the mobile phase was helium. Polar compounds, containing protic hydrogenes were derivatized with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), thus converted to the corresponding trimethylsilyl derivatives and analyzed by GC-MS.

3.16. Extraction of proteins from *Hypericum calycinum* cell cultures

All steps of enzyme extraction were applied at 4 °C. The treated cells were collected by filtration at three-hour-intervals after induction. Six grams of cells were homogenized for 10 minutes with 0.6 g Polyclar AT, 5 ml potassium phosphate buffer (Kpi) (2.13.1) pH 7 containing 1 mM dithiothreitol (DTT) and sea sand. The homogenate was centrifuged for 10 minutes at 9000 rpm. The soluble proteins were present in the supernatant.

3.17. Gel filtration chromatography

This technique is applied to separate molecules in one solution due to their size. A PD_{10} column (Pharmacia, Freibug, Germany) was used to remove the low molecular mass substances from the proteins after cell extraction. The PD_{10} column was equilibrated at first with 25 ml potassium phosphate buffer pH 7 (2.13.2), then 2.5 ml from the extracted supernatant (3.16) were added to the column and the protein fraction was eluted from the column by adding 3.5 ml potassium phosphate buffer. The column was washed after that with 25 ml NaOH 0.16 M to regenerate the column followed with distillated water till pH be neutral (2.13.5).

3.18. Determination of protein concentration (Bradford Assay)

Protein concentration was determined according to Bradford (Bradford, 1976) by using Coomassie Brilliant Blue G 250. By mixing with the protein the absorbance maximum of this solution will shift from 465 nm to 595 nm, which is measured by using UV/Vis spectrophotometry. To measure the protein concentration of one sample, add 900 µl Bradford reagent (2.13.3) with 95 µl dH_2O and 2 µl from the protein sample, mix gently, incubate for 5 minutes at room temperature and measure the absorbance at 595 nm. The protein concentration was calculated from a calibration curve, which was established using 1 to 10 µg/ml bovine serum albumin (BSA) as standard.

3.19. Protein storage

Protein can be stored for further usage by mixing gently with 50% autoclaved glycerin and keeping at – 20 °C. In this way the solution will not be frozen and the protein stays for about three weeks without marked loss of activity.

3.20. Isolation of mRNA

The isolation of mRNA was performed by using the mRNA extraction kit from GE Healthcare. The principe is based on the addition of guanidinium thiocyanate (GTC), an chaotropic agent which inhibits the activity of RNases, and on the affinity of the mRNA poly (A) tail to cellulose oligo (dT)-matrix. As per manufacturer's instruction, 100 mg cultured cells of *H. calycinum* before and after elicitor treatment was ground with liquid nitrogen and extracted in buffer containing high amount of GTC. The extract was diluted three times with buffered solution to precipitate the proteins, to reduce the concentration of GTC and to allow the mRNA poly (A) tail to bind to the cellulose oligo (dT)-matrix. After few steps of washing with high-salt and low-salt buffer, the mRNA was precipitated by adding ethanol and potassium acetate and eluted with RNase-free water. Purified mRNA was used either directly for reverse transcription after determination of the concentration or stored at – 80 °C.

3.21. Isolation of total RNA

The RNeasy plant mini kit (Qiagen) was used to isolate the total RNA from induced and non-induced cell cultures of *H. calycinum.* One hundred mg of either freshly harvested cells or cells stored at – 80 °C were ground with liquid nitrogen and transferred to lysis buffer containing GTC to inactivate the RNases. After few steps of homogenization and cells lysis, the lysate was transferred to a RNeasy Mini spin column. The RNA binds to a silica-gel-based membrane in presence of ethanol and after washing, RNA was eluted with RNase-free water, the concentration was determined and the RNA was stored at – 80 °C for further usage.

3.22. RNA agarose gel electrophoresis

Total RNA was separated in agarose gels during the run towards the anode in an electric field. About 40 ml gel was prepared by dissolving 0.48 g agarose in 37.84 ml 1x MOPS buffer (2.13.11) by boiling for short time in a microwave. After cooling down to 60 °C, 2.16 ml formaldehyde 37% was added and then ethidium bromide at a final concentration of 0.5 µg/ml. The solution was poured into a gel tray. Prior to running the gel, it was equilibrated in RNA running buffer (2.13.11) for at least 30 min. All the steps involving the work with ethidium bromide and formaldehyde should be done under fume hood.
The samples (8 µg) were mixed with 5x RNA loading buffer (2.13.11) 4:1 (v/v), incubated for 3-5 minutes at 65 °C, chilled on ice and loaded into the equilibrated RNA gel, which was run for 30 min in a gel chamber including RNA running buffer at a voltage of 60 V. A DNA marker was run in parallel to compare the size of the separated RNA fragments. The 28S and 18S rRNA bands were visualized in the gel by using an imager under UV light at 302 nm.
Electrophoresis tanks should be cleaned with 0.5% SDS, thoroughly rinsed with RNase-free water and then rinsed with ethanol and allowed to dry. Water and other solutions should be treated with 0.1% diethylpyrocarbonate (DEPC) as inhibitor of RNases as follows: add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously, incubate the solution overnight at 37 °C and autoclave to remove any trace of DEPC.

3.23. Northern blot

A Northern blot is used to prove if a gene is expressed by detecting its mRNA in the sample. Total RNA was isolated from 100 mg *H. calycinum* cell cultures every three hours after elicitation with yeast extract according to the RNeasy Plant Mini Kit instructions (Qiagen). After measuring the concentration (3.5), eight µg total RNA from each sample was separated in an agarose gel including formaldehyde (3.23). The probe was prepared by amplifying 300 bp of BPS cDNA by using a specific pair of primers (Prob BPS fwd and Prob BPS rev) (2.12). As control another 300 bp probe of *H. androsaemum* CHS cDNA was also prepared by using a specific pair of primers (Prob CHS fwd and Prob CHS rev) (2.12) to be sure that no cross hybridization happened and the expressed gene was *BPS*.

The samples were transferred to a positively charged nylon membrane (Roth) by a capillary blotting system (Fig. 3.1). Labeling, pre-hybridization, hybridization, washing and detection were done according to the instructions of the DIG-High Prime DNA kit (Qiagen).

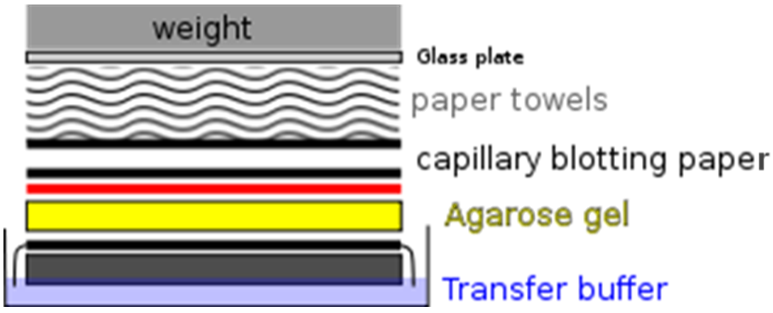


Fig. 3.1. Capillary blotting system setup for the transfer of RNA from an electrophoresis gel to a blotting membrane (from Wikimedia Commons)

3.24. Reverse transcription

Reverse transcription forms single-strand DNA from a mRNA or total RNA template by reverse transcriptase, which is an RNA-dependent DNA polymerase and used to catalyze the oligo-dT primed synthesis of first strand cDNA (DNA complementary to the respective mRNA).
The following components were mixed in a PCR-tube and incubated for 10 minutes at 70 °C.

3'-RACE-cDNA		5'-RACE-cDNA	
Poly(A)-RNA	1 µg	Poly(A)-RNA	1 µg
3'-CDS Primer	1 µl	Smart II oligo Primer	1 µl
10 mM dNTP Mix	1 µl	5'-CDS Primer	1 µl
Nuclease free water	ad 10 µl	10 mM dNTP Mix	1 µl
		Nuclease free water	ad 10 µl

The following components were added to both tubes:

5x Buffer	4 µl
0.1 M DTT	2 µl
Reverse transcriptase	1 µl
RNase inhibitor (40 U/µl)	0.5 µl
Nuclease free water	2.5 µl

The new mixtures were incubated at 37 °C for 50 minutes. The newly formed cDNA was either directly amplified to double-strand DNA by using gene specific primers in a standard PCR or stored at – 20 °C for further usage.

3.25. Plasmid construction

DNA fragments such as PCR products can be ligated to cloning vectors by using T4-DNA-ligase, where the 5'-phosphate group of one DNA end binds the 3'-OH group on the other DNA end forming phosphodiester bond. Requirement is the complementary of the ends of vector with the ends of the insert. By using T-vectors that have a single thymidine (T) overhang at each 3' end, DNA fragments amplified with *Taq* DNA polymerase can be easily ligated to the vector. The *Taq* DNA polymerase adds usually a deoxyadenosine (A) to the

3' end of PCR product. For other ligations using vectors such as pRSET B, the ends of the DNA fragments were digested by restriction enzymes, so that sticky ends were created. These fragments have to be amplified by using primers including the restriction sites of these enzymes (2.12). The restriction sites are part of the vector MCS and should be absent from the sequence of the DNA fragment. In this work the ends of the DNA fragment were digested with *Nhe*I and *Kpn*I and then ligated into the *Nhe*I/*Kpn*I linearized expression vector pRSET B.

The following ligation protocol was used in the work:

Digested vector	1 µl
Digested DNA fragment	6 µl
10x ligation buffer	1 µl
T4-DNA-Ligase (5U/µl)	0.5 µl
dH_2O	ad 10µl

The mixture was incubated either for 3-4 hours at room temperature or overnight at 4 °C.
The product was transferred to DH5α (3.29), the DNA was isolated (3.31) after selection (3.30) and restricted with the appropriate restriction enzymes (3.32).

3.26. Preparation of competent cells

E. coli cells are able to take up an extracellular DNA after being treated with $CaCl_2$; calcium ions increase the permeability of the cell membrane to pass a foreign DNA. Competent cells were prepared according to Mandel and Higa (1970). One colony of *E. coli* from an agar plat was picked up and grown overnight in 5 ml liquid LB medium (2.8.3) at 37 °C with shaking at 225 rpm. One ml from the new culture was transferred to 50 ml liquid LB medium until the bacterial culture reached an optical density of 0.6-0.8 at 600 nm, cells were cooled on ice for 10 min and centrifuged at 4 °C and 3000 rpm for 10 min. The bacterial pellet was resuspended carefully in 25 ml ice-cooled 0.1 M $CaCl_2$. After 10 min incubation on ice, the cells were recovered by centrifugation at 4 °C and 3000 rpm for 10 min. The supernatant was discarded. The pellet was resuspended carefully in 5 ml 0.1 M $CaCl_2$ containing 15% glycerin.

Then the cell suspension was kept at 4 °C for 19-24 h to achieve high competency. Afterwards the competent cells were stored at – 80 °C as 50 µl aliquots until further use.

3.27. Determination of the optical density (OD)

The bacterial growth was determined by measuring the turbidity at the wavelength 600 nm by using a UV/Vis spectrophotometer. The solution without bacteria was used as blank. One unit absorbance value at wavelength 600 nm corresponds to about 8 x 10^8 cells (Bertram and Gassen, 1991).

3.28. Storage of bacteria

The bacterial cultures were stored either for one month at 4 °C on solid medium (LB for *E. coli* and YEB for *Agrobacterium*) with appropriate antibiotics, or for long time at – 80 °C by mixing the bacterial solution with 20% (v/v) LB/YEB-glycerin mixture.

3.29. Transformation into *E. coli* DH5α

At first 50 µl *E. coli* DH5α competent cells from a – 80 °C storage (3.26) were incubated 10 minutes in ice, immediately followed by addition of 5 µl foreign plasmid and incubation of the mixture at 4 °C for 30 minutes. Heat shock at 42 °C for 45 seconds was applied, followed directly by 5 minutes incubation at 4 °C to transfer the DNA into the cells. The cells were grown in 250 µl SOC medium by shaking for one hour at 37 °C, spread on a plate of solid LB medium (2.8.3) containing 100 µg/ml ampicillin in case of T and pRSET B vector or 50 µg/ml kanamycin in case of pBIN19/BPS vector (2.3) and incubated overnight at 37 °C.

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3.30. Selection of positive recombinants

The addition of appropriate antibiotic during the plasmid transformation into *E. coli* and on the agar plate after the transformation is an important step to select the transformed and non-transformed cells, especially when transforming plasmids that do not have the *Lac*Z gene such as pRSET B. For the transformation of plasmids including *Lac*Z gene (encodes *β*-galactosidase) such as T-vector, other components were added to the agar plate after the transformation (2.13.6). The components 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-Gal) and isopropyl *β*-D-1-thiogalactopyranoside (IPTG) (2.13.6) allow a blue/white screening to detect the recombinant colonies. Blue/white selection is based on the ability of *β*-galactosidase to cleave the substrate X-gal to give galactose and an insoluble blue product. The multiple cloning site of this vector is embedded in the coding region of the *lac*Z gene and when the insertion successfully happens, the *Lac*Z gene will not be able to form *β*-galactosidase and white colonies on the plate will appear. In case of unsuccessful insertion, blue colonies will appear, that contain the original plasmid without interruption of *Lac*Z gene by foreign DNA.

3.31. Plasmid isolation from *E. coli*

One colony from the overnight-growing plate was transferred into 5 ml liquid LB medium (2.8.3) containing the appropriate antibiotic. The bacterial culture was incubated overnight by shaking at 140 rpm at 37 °C. The next day 2 ml from the new culture were harvested by centrifugation for 5 minutes at 5000 rpm. The bacterial pellet was then resuspended in 300 µl buffer 1 (2.13.8). Then 300 µl buffer 2 (2.13.8) were added to the bacterial suspension to lyse the cells and incubated on ice for not more than five minutes followed by 300 µl buffer 3 (2.13.8). Samples were mixed gently and incubated on ice for 20 minutes. The solution was centrifuged for 15 minutes at 13000 rpm. The clear supernatant was transferred to a new reaction tube and extracted with an equal volume of chloroform. The DNA was precipitated from the aqueous phase by adding 2/3 volume of 2-propanol and centrifuged at 13000 rpm for 30 minutes. The pellet was washed with 500 µl 70% ethanol and the purified plasmid was dried and redissolved in 50 µl dH_2O. This plasmid can be directly used after measuring the concentration or stored at – 20 °C for further usage.

3.32. Standard restriction reaction

DNA restriction endonucleases are enzymes that recognize a particular sequence of DNA bases and catalyze the cleavage of the double stranded DNA. Plasmid restriction was applied using restriction endonucleases either to check whether the ligation of a DNA fragment into the cloning vector was successful due to the restricted fragment size after running in an agarose gel or to allow for one insert to be ligated into one circular vector after opening it by using appropriate restriction enzymes.

The following restriction protocol was used in the work:

Plasmid DNA	1 µg
10x restriction buffer (depends on the enzyme)	2 µl
Restriction enzyme/s	0.5 µl
dH_2O	ad 20 µl
Reaction was incubated at 37 °C for 2 h	

3.33. DNA sequencing

The isolated DNA from *E. coli* (3.31) was sent for sequencing to MWG, Germany. Sequencing reactions were also carried out in our laboratory by using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The DNA nucleotides were determined by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). This kit contains *Taq* DNA polymerase and the four ddNTPs. Each one of the four dideoxynucleotide chain terminators is labeled with a specific fluorescent dye, to give different wavelengths of fluorescence and emission. The DNA sequencer analyzes automatically DNA molecules labeled with multiple fluorescent dyes. All sequences were edited and analyzed with the Lasergene 99 DNASTAR Software. The primers (T7, SP6) and (pRSET B fwd, pRSET B rev) (2.12) were used for the sequencing.

The sequencing reaction was as follows:

BigDye solution	2 µl
DNA template	700 ng
Primer	1 µl
dH_2O	ad 10 µl

The thermocycler program was as follows:

1. Denaturation	15 seconds at 95 °C	
2. Annealing	25 seconds at 50 °C	30 cycles
3. Elongation	1 minute 30 s at 72 °C	

3.34. Sequencing gel

The sequencing gel (2.13.9) was prepared by dissolving 9 g urea in 30% acrylamide solution, 10x TBE buffer and dH_2O. Then 10 µl TEMED and 230 µl 10% APS were added. The solution was poured between two glass plates. The addition of APS and TEMED started the polymerization. After 1 hour the components of the sequencing were loaded onto the gel.

3.35. Analysis of DNA data

DNA sequences and deduced amino acid sequences were analyzed using programs such as EditSeq, MegAlign and SeqMan from the software DNASTAR. The NCBI BLAST (Basic Local Alignment Search Tool) was used for a database comparison with known DNA and amino acid sequences. This program is accessible at: www.ncbi.nlm.nih.gov/BLAST/.

3.36. Transformation into *E. coli* BL21 (DE3) pLysS

Plasmid-containing DNA for heterologous protein expression (pRSET B) was transformed into the expression host *E. coli* BL21 as follows: 50 µl *E. coli* BL21 competent cells from a – 80 °C storage were incubated for 10 minutes on ice. Then 5 µl expression vector were added immediately and the mixture was incubated at 4 °C for 30 minutes. Heat shock at 42 °C for 20 seconds was applied to open the competent cells, followed directly by 5 minutes incubation at 4 °C to ensure its closure. The cells were then grown in 700 µl SOC medium (2.8.3) by shaking for one hour at 37 °C. Cells were harvested by centrifugation for 10 minutes at 5000 rpm, the supernatant (500 µl) was discarded and the pellet was resuspended in the rest of the solution. About 80 µl of bacterial suspension was spread on a plate of solid LB medium containing ampicillin (100 µg/ml) and chloramphenicol (60 µg/ml) and incubated overnight.

3.37. Expression of recombinant protein

A single bacterial colony from a LB agar plate (3.36) was transferred into 10 ml liquid LB medium (2.8.3) containing ampicillin (100 µg/ml) and chloramphenicol (60 µg/ml) and incubated overnight with shaking at 225 rpm at 37 °C. Four ml of the overnight culture were transferred into 100 ml LB medium and incubated with shaking at 225 rpm at 37 °C. Once the culture reached the OD_{600} 0.6-0.8, recombinant plasmid was induced by addition of isopropyl -*β*-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and the culture was incubated overnight at 25 °C with shaking at 150 rpm in the dark.

3.38. Extraction and purification of the recombinant protein

The bacterial suspension was centrifuged at 5000 rpm and 4 °C for 20 minutes and the pellet was resuspended in 3 ml ice-cold lysis buffer (2.13.7). Sonication of the cells was carried out on ice-cold water for 5 min at 50% pulses using a Branson Sonifier B15 (Heinemann, Schwäbisch Gmünd, Germany). The resulting solution was centrifuged at 9000 rpm and 4 °C for 20 min.

The resulting supernatant including the desired protein was purified by using the nickel-nitrilotriacetic acid (Ni-NTA) protein purification system (3.39). Two hundred µl Ni-NTA were added to 3 ml extracted supernatant and the mixture was incubated with shaking at 4 °C for 1 hour and added into an empty PD_{10} column but has a filter at the bottom. The mixture was washed four times with 1 ml washing buffer (2.13.7) and the His_6-tagged-fusion protein was eluted with 3.5 ml elution buffer (2.13.7). Imidazole was removed from the eluate by gel filtration chromatography (3.17) using a PD_{10} column equilibrated with 0.1 M potassium phosphate buffer (Kpi) pH 7 (2.13.2).
The concentration of the pure protein was measured due to Bradford (3.18). The purity and the size were detected by SDS-PAGE (3.41). This protein was either directly used for the protein characterization (3.44) or stored at – 20 °C for further usage (3.19).

3.39. Affinity chromatography on Ni-NTA

The principle of the affinity chromatography is based on the specific interaction between the tagged protein wished to be purified and the affinity matrix, so that the overexpressed protein binds specifically to the matrix while the other proteins are washed away and the target protein will be eluted thereafter with a special buffer, because the matrix has more affinity to imidazole than to the His-tag. In this work the BPS cDNA was cloned to the pRSET B vector (3.25) and the protein was purified by using the Ni-NTA system (3.38).
The Ni-NTA system can purify 6x His-tagged recombinant fusion proteins because of the affinity interaction between Ni^{2+} ions on a matrix and the histidine side chain, followed by washing away foreign proteins from the matrix. The 6x His-tagged fusion proteins can be eluted by adding buffer containing high amount of imidazole (2.13.7).

3.40. Precipitation of protein by DOC and trichloroacetic acid

This method provides rapid precipitation of low protein concentrations. One volume of protein solution (about 20 µg) was mixed with 1/10 volume of deoxycholate (DOC) solution (2.13.4). DOC forms complexes with proteins to enhance precipitation at low pH. Then, one volume of the previous solution was mixed with 1/10 volume of trichloroacetic acid (TCA) solution (2.13.4). TCA is used for precipitation of DOC-protein complexes. Then the solution was mixed and incubated overnight at 4 °C. The precipitated protein was centrifuged for

15 min at 4 °C and 13000 rpm. The supernatant was disposed and the pellet (concentrated protein) was re-suspended in 10 µl 1.5 M Tris-HCl pH 8.8.

3.41. SDS-PAGE

This technique is used to separate proteins according to their molecular masses while migration towards the anode in polyacrylamide gel electrophoresis. It is also used to confirm the successful expression of cloned cDNAs in heterologous expression systems (Laemmli, 1970). Proteins (10 µg) became negatively charged by adding SDS and were denatured by heating them with 1:1 protein loading buffer (2.13.10) for five minutes at 95 °C. The polyacrylamide gel is formed by polymerizing the monomer acrylamide at room temperature with APS and TEMED.

Two gels were prepared; the first one is the separating gel 12% (2.13.10) which allows highest resolution of separation of proteins between 10 and 200 kDa. It was poured in the gel casting between two glass plates followed by a thin layer of water, which was removed after polymerization. The second gel is the stacking gel (2.13.10), which should be poured over the separating gel directly after the water was removed to avoid gel drying. The samples were loaded into the wells of the stacking gel in parallel with a protein marker for determination of the relative molecular weights of the separated proteins. The gel running was for about 45 minute at 25 mA in the stacking gel and 35 mA in the separating gel and 200 V supplied by a BioRad power supply. The gel was then incubated overnight in the staining solution (2.13.10), followed by destaining solution (2.13.10) until the separated proteins were clearly visible in the gel.

3.42. Enzyme assay

The benzophenone synthase assay was performed in a final volume of 250 µl (2.13.2) containing 9.9 µM benzoyl-CoA as starter substrate and 18.1 µM malonyl-CoA as extender molecule (Table 3.1). The mixture was incubated for 20 minutes at 35 °C. The reaction was stopped by adding 20 µl of 3 M trichloroacetic acid. The products formed were extracted twice with 250 µl ethyl acetate and centrifuged at 13000 rpm for 10 min. The organic phase was dried under vacuum. The residue was dissolved in 50 µl HPLC grade methanol.

A sample with denatured enzyme served as negative control by boiling the enzyme for 10 minutes. Analysis of the enzymatic products was performed by HPLC.

Table 3.1. Benzophenone synthase assay

	Stock solution	Used amount	Final concentration
Benzoyl-CoA	0.23 mM	10 µl	9.9 µM
Malonyl-CoA	0.47 mM	10 µl	18.1 µM
Enzyme			2 µg pure protein or 20-100 µl crude extract
Potassium phosphate buffer pH 7	0.1 M	ad 250 µl	

3.43. Characterization of benzophenone synthase

3.43.1. Determination of pH and temperature optima

The enzyme assays were performed as described in (3.42) with different pH values from 5.5 to 8.5 and incubated for 10 minutes at 35 °C. Then the enzymatic products were analyzed by HPLC and the optimum pH was determined. At the optimum pH value, another series of incubations were performed at different temperatures between 15 to 45 °C.

3.43.2. Determination of the DTT concentration optimum

The measurement of the optimum DTT concentration was performed as described in (3.42) where DTT concentrations between 0 – 100 µM were added. After 10 min incubation at 35 °C, the products were extracted and analyzed by HPLC.

3.43.3. Study of substrate specificity

At the pH and temperature optima, BPS assays were performed using malonyl-CoA as extender substrate and a series of starter substrates: benzoyl-CoA, *o*-hydroxybenzoyl-CoA, *m*-hydroxybenzoyl-CoA, *p*-coumaroyl-CoA, acetyl-CoA, butyryl-CoA, isobutyryl-CoA, isovaleryl-CoA, hexanoyl-CoA and octanoyl-CoA. After incubation at 37 °C for 10 minutes in Kpi buffer (2.13.2) pH 7, the products were extracted and analyzed by HPLC.

3.43.4. Determination of kinetic parameters

The kinetic properties of BPS were determined by using different concentrations of benzoyl-CoA (0.49-19.8 µM) and a saturating concentration of malonyl-CoA, or using different concentrations of malonyl-CoA (0.9-36.2 µM) and a saturating concentration of benzoyl-CoA. The extracted products were analyzed by HPLC. The K_m values were calculated by the program Hyper 32.

4. Results

4.1. Transformation of *Hypericum perforatum via Agrobacterium*

One aim of this work was to find a protocol for the transformation of *H. perforatum* using *Agrobacterium.* Various conditions had to be established before starting the transformation procedure.

4.1.1. Establishment of transformation conditions

4.1.1.1. Use of the binary vector pBIN19/BPS for transformation

The BPS ORF from *H. androsaemum* was previously cloned into the binary vector pBIN19 (Fig. 4.1), yielding the plasmid pBIN19/BPS (Feye, 2007). The plasmid pBIN19/BPS was transferred into the *A. tumefaciens* strain C58C1 to be used for the transformation. The same plasmid was used as a positive control to detect the transformation efficiency *via* PCR. Successful transformation was examined *via* amplification of the target gene in comparison to the band size amplified from the pBIN19/BPS plasmid (Fig. 4.2). PCR was done using the primers *lac*Z fwd and *lac*Z rev (2.12) and confirmed by *Hind*III restriction.

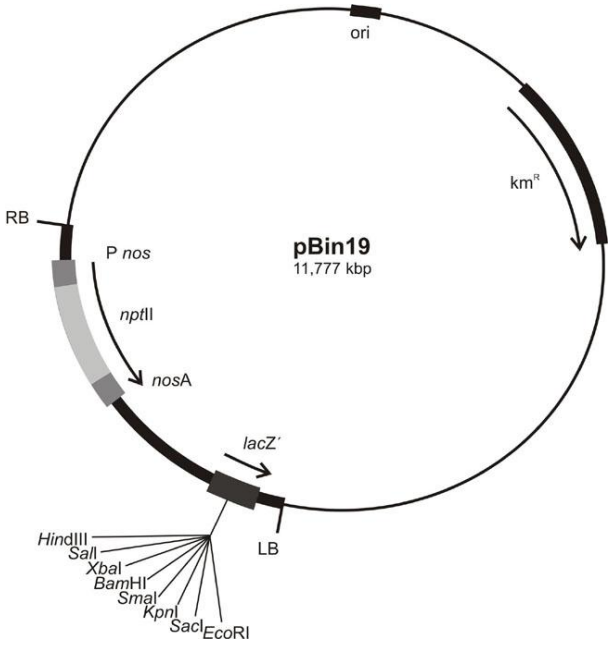


Fig. 4.1. Binary vector pBIN19 (Bevan, 1984) containing right and left borders (RB and LB), a multiple cloning site (MCS), the *Lac*Z gene coding for *β*-galactosidase and the neomycin phosphotransferase gene (*npt*II)

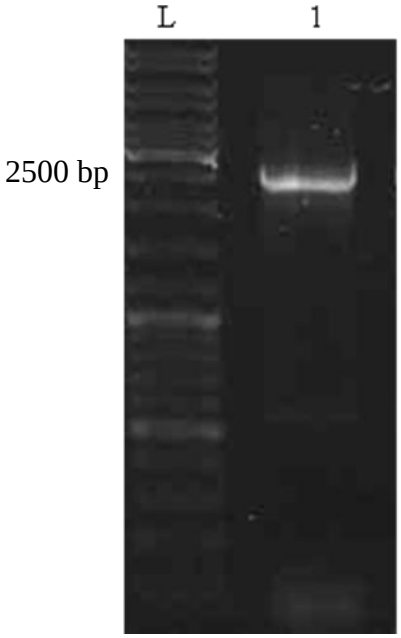


Fig. 4.2. Amplification of the *Lac*Z gene containing the BPS ORF from the vector pBIN19/BPS. L: DNA ladder mix (Fermentas), 1: Amplified target construct from the plasmid pBIN19/BPS

4.1.1.2. Seed germination

Surface-sterilized seeds were cultivated in solid hormone-free Murashige Skoog (MS) medium (2.8.2) at 25 °C for a 16 h light/8 h dark cycle. Three-week-old seedlings were transferred to fresh medium and grown under the same conditions. After 6-7 weeks, the plants were completely developed (Fig. 4.3). The medium was refreshed every 7-8 weeks.

Fig. 4.3. *Hypericum perforatum in vitro* plant

4.1.1.3. Establishment of a *Hypericum perforatum* regeneration system

The regeneration ability of various untransformed parts of *H. perforatum* was studied. Changing the hormonal composition was a target to find best conditions for regeneration of explants (Table 4.1).

The best medium was MS (solid) containing 0.5 mg/l 6-benzylaminopurine (BAP) and 1 mg/l indole-3-acetic acid (IAA) (2.8.2). This medium was able to produce reasonable amounts of new shoots within four weeks from most of the leaves, stems and roots tested (Fig. 4.4). No medium was able to produce new shoots from flowers or fruits.

Table 4.1. Regeneration media used for *Hypericum perforatum*

Medium	Phytohormones	Regeneration efficiency
MS	0.5 mg/l BAP + 1 mg/l IAA	High shoot regeneration frequency
	1 mg/l BAP + 2 mg/l NAA	Few shoots from the explants
	1 mg/l BAP + 0.1 mg/l NAA	No regeneration
	1 mg/l kinetin + 0.5 mg/l NAA	No regeneration

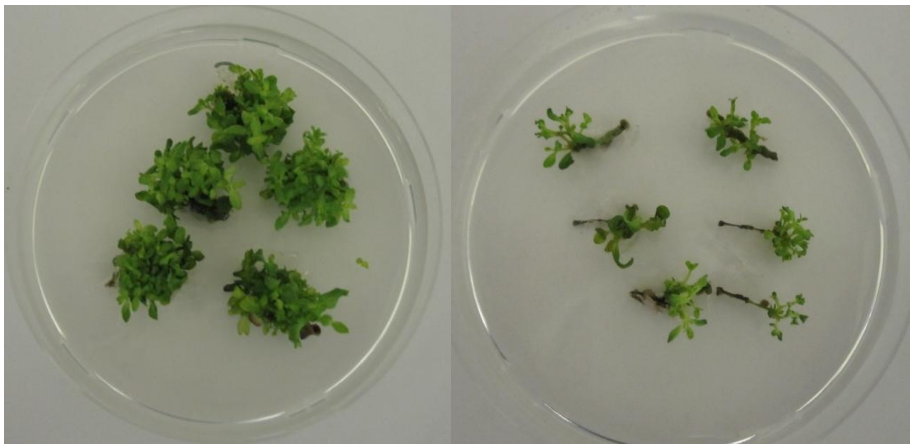


Fig. 4.4. Regeneration of *Hypericum perforatum* from leaves (left) and stems (right)

4.1.1.4. Kanamycin effect on *Hypericum perforatum*

Kanamycin is an aminoglycoside antibiotic. It can be used as an agent for selection during transformation because of its ability to stop the growth of untransformed plants and to kill them afterward (Fig. 4.5). Different concentrations of kanamycin were added to the *H. perforatum* growing medium (MS) to study the effect of the antibiotic on the growth of untransformed plants. It was found that kanamycin concentrations lower than 50 mg/l took at least 4 weeks to stop the growth of well-grown plants (6 to 8-week-old). However, higher concentrations between 100-200 mg/l were able to stop plant growth within 10 days.

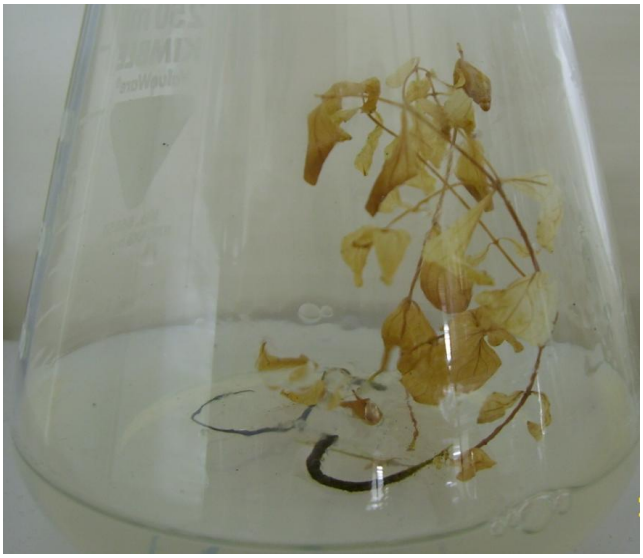


Fig. 4.5. Effect of kanamycin on *Hypericum perforatum in vitro* plants

4.1.2. Variations of the transformation procedure

4.1.2.1. *Agrobacterium tumefaciens*-mediated transformation of *Hypericum perforatum*

The *A. tumefaciens* strain C58C1 containing the binary vector pBIN19/BPS was used to transfer the T-DNA that contained the BPS ORF and the neomycin phosphotransferase gene (*npt*II) into *H. perforatum* (Helos, Topas and Elixir). Leaf and stem explants were used as plant material for the transformation. They were co-cultivated with *A. tumefaciens* (3.2.2). Thereafter, the explants were transferred to antibiotic-free MS solid regeneration medium (2.8.2). It was noticed that there was no over-growth by *Agrobacterium* on the medium, and the explants started to regenerate after three weeks. Complete plants were developed within 6 weeks.

Eight-week-old shoots were transferred to MS medium including 50 mg/l kanamycin to select the supposed transformed shoots. However, kanamycin did not stop the growth within short time, suggesting successful transformation. Some of the surviving shoots were collected and their genomic DNA was isolated according to the protocol of DNeasy Plant Mini Kit (Qiagen). PCR was done using *Taq* DNA polymerase, gene-specific primers (*lac*Z fwd and *lac*Z rev) and genomic DNA from regenerated shoots as a template (2.12). The rest of the

shoots was transferred to solid MS medium containing 100 mg/l kanamycin. This concentration caused all regenerated shoots to die within 14 days.
No specific PCR product was obtained when genomic DNA from a number of regenerated shoots was used as a template, indicating unsuccessful transformation. As a control the target gene was successfully amplified from the binary vector pBIN19/BPS used as template.

4.1.2.2. High concentration of *Agrobacterium*

To overcome the plant defense mechanism against the bacteria, 200 ml YEB medium containing a well-grown *Agrobacterium* suspension (OD = 0.8) was prepared. The explants were co-cultivated for one hour in this medium and then transferred to the antibiotic-free regeneration medium. Cefotaxime (250 mg/l) was added to the medium after two days to get rid of the bacteria. Necrosis in the explants was induced as a defense response. The explants became dark brown in colour and died after 10 days.

Further trials (4.1.2.3) were done to optimize the transformation conditions in such a way that *Agrobacterium* survives the plant resistance.

4.1.2.3. Optimization of transformation conditions

Co-cultivation was carried out as previously described (4.1.2.2) but with shortening its duration (2, 5 and 10 minutes). After transfer to regeneration medium, over-growth by *Agrobacterium* was observed after one week and the shoots started to grow within three weeks. Cefotaxime (250 mg/l) was added to the medium to get rid of the bacteria. A low concentration of kanamycin (1, 2, 5 and 10 mg/l) was added after five days to apply early selection on the explants (Fig. 4.6).
The low concentration of kanamycin (5 mg/l) was able to stop the regeneration of the control explants (without incubation with *Agrobacterium*) but not the supposed transformed plant cells (Fig. 4.6).
DNA was isolated from the shoots that still grew. PCR was applied to check for successful transformation. However, no specific PCR product was obtained.

Fig. 4.6. Early selection applied on leaf, stem and root explants using 5 mg/l kanamycin after incubation with *Agrobacterium tumefaciens*

While aerial parts of *H. perforatum* are hypericin and hyperforin-rich organs, seedlings or underground organs contain only traces of these antibacterial compounds (Soelberg *et al*., 2007; Zobayed *et al*., 2006). Thus, alternative protocols used seedlings and roots as explants for transformation. This strategy was expected to avoid the antibacterial effect exhibited by the above compounds.

4.1.2.4. Transformation of roots

Root parts (primary root, secondary roots and root tip) from three varieties of *H. perforatum* (Helos, Topas and Elixir) were isolated and incubated for short time periods with *A. tumefaciens*, as described previously (4.1.2.3). Regeneration of shoots occurred; however, the isolated DNA samples from the supposed transgenic shoots gave negative PCR results.

4.1.2.5. Seedlings as material for transformation

As mentioned above, the benefit of the usage of seedlings for transformation is the relatively low concentration of hyperforin and hypericin compared to that of well-grown upper parts of *H. perforatum*.
This property provided a chance for *Agrobacterium* to survive during co-cultivation and to transfer the T-DNA into the plant cells. Seedlings were collected and co-cultivated for two minutes with *A. tumefaciens*. No regenerated shoots were detected after the selection process using 5 mg/l kanamycin.

To examine the response of other plant parts in transformation, fruits and flowers as well as callus cultures were used (4.1.2.6).

4.1.2.6. Infection of fruits, flowers and callus

Intact fruits and flowers did not regenerate shoots under the conditions described above (4.1.1.3). Thus, they could not be used as material for transformation. On the other hand, the callus was able to produce shoots. These started to grow three months after induction. Nevertheless, they did not show any resistance to kanamycin (5 mg/l) after the infection.

4.1.2.7. Transformation using the high-virulent strain AGL1

AGL1 is a hyper-virulence strain of *A. tumefaciens*, it contains the binary vector UGAB7 including the *GUS* gene, which codes for the enzyme *β*-glucuronidase to be used as a reporter for transformation. This strain carries a resistance to bialaphos, which has herbicide properties. After co-cultivation of explants from different parts of *H. perforatum* plants (Helos, Topas and Elixir) with *Agrobacterium* AGL1, they were transferred to the regeneration medium containing cefotaxime (250 mg/l) to get rid of the bacteria and bialaphos (5 mg/l) to make selection pressure (3.2.3). No resistant shoots developed.

4.1.2.8. Vacuum infiltration

This technique may improve the transformation efficiency and allow the penetration of the bacteria into the intercellular spaces by replacing air with medium under reduced pressure during co-cultivation. Most of the trials were done with leaves, as described in (3.2.5), because of the high number of stomata compared to other organs of the plant. The conditions of this process are critical because vacuum can damage the leaves after too long exposure period. The explants were transferred to the favorite regeneration medium (2.8.2) containing kanamycin (5 mg/l) for selection. No regeneration was achieved.

After failure of the transformation trials using *A. tumefaciens*, it was advisable to use *A. rhizogenes*, another species of the genus *Agrobacterium*, which can induce growth of hairy roots from infected cells.

4.1.2.9. *Agrobacterium rhizogenes*-mediated transformation of *Hypericum perforatum*

The *A. rhizogenes* strain LBA 1334 containing the vector pRi 1855 is not disarmed. This strain is able to produce hairy roots from infected plant cells by transferring the T-DNA containing auxin and cytokinin genes. This property avoids the selection by antibiotic or herbicide after the infection and helps to determine the efficiency of the transformation by monitoring the growth of hairy roots from the infected cells. It also facilitates the isolation of DNA from the newly formed roots and the detection of the transferred gene by PCR. The *A. rhizogenes* strain was grown and prepared for transformation as described previously (3.2.4). Unfortunately, no hairy roots could be induced from leaves. Similarly, shoots and roots were used without any successful transformation.

4.2. Benzophenone synthase (BPS) from *Hypericum calycinum* cell cultures

The second aim of this work was to detect xanthone formation induced upon elicitation with yeast extract in cell cultures of *H. calycinum* and to use this system for cloning of a BPS cDNA and characterization of the recombinant protein.

4.2.1. Growth and elicitation of the cell cultures of *Hypericum calycinum*

H. calycinum cell cultures were grown at 25 °C in the dark in liquid LS medium (2.8.1) and transferred into 50 ml fresh medium every 14 days starting with 3 g cells. Their growth was monitored over 14 days. It was noticed that the cell mass lost some weight in the first three days, before the fresh weight started to increase linearly till reaching a plateau after 11 days (Fig. 4.7).

Elicitors such as chitosan (25 mg/l), methyl jasmonate (100 µM) and yeast extract (3 g/l) were added separately to the cell cultures of *H. calycinum* on the fourth day after sub-cultivation. Yeast extract was the only elicitor that could stimulate the cells to produce xanthones. The final concentration was 3 g/l. Nine hours after elicitation, cells gained a dark yellow colour (Fig. 4.8). HPLC analysis of cell-free methanolic extracts showed one major peak which was absent from the control (non-treated) cells (Fig. 4.9) and analyzed as follows:

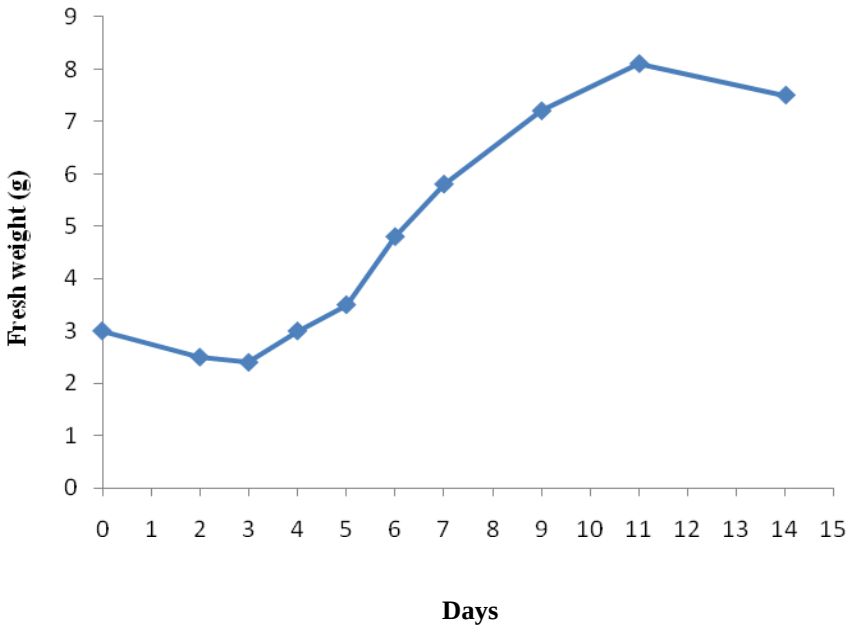
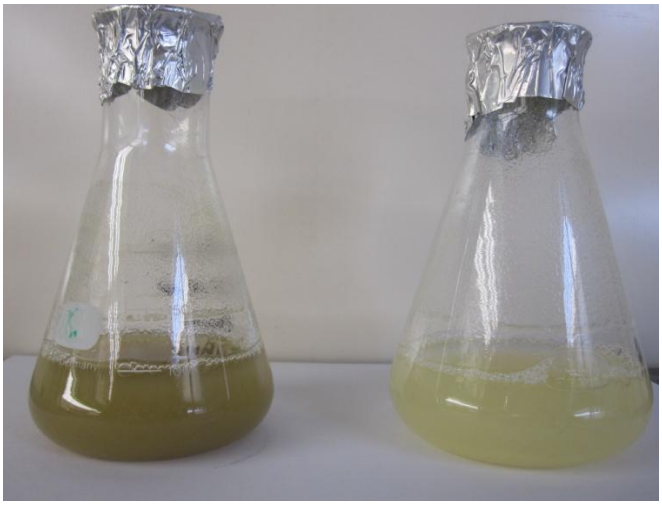


Fig. 4.7. Growth of *Hypericum calycinum* cell cultures in liquid LS medium in the dark. Data are mean values of two independent experiments



A **B**

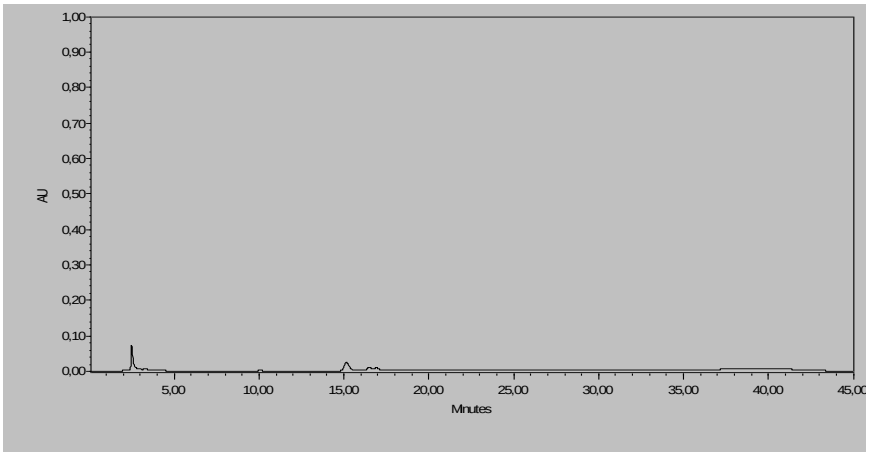
Fig. 4.8. *Hypericum calycinum* cell cultures in LS medium
A: Nine hours after addition of yeast extract
B: Before treatment

4.2.2. Analysis of 1,3,6,7-tetrahydroxy-8-prenylxanthone by HPLC, ESI-MS and GC-MS

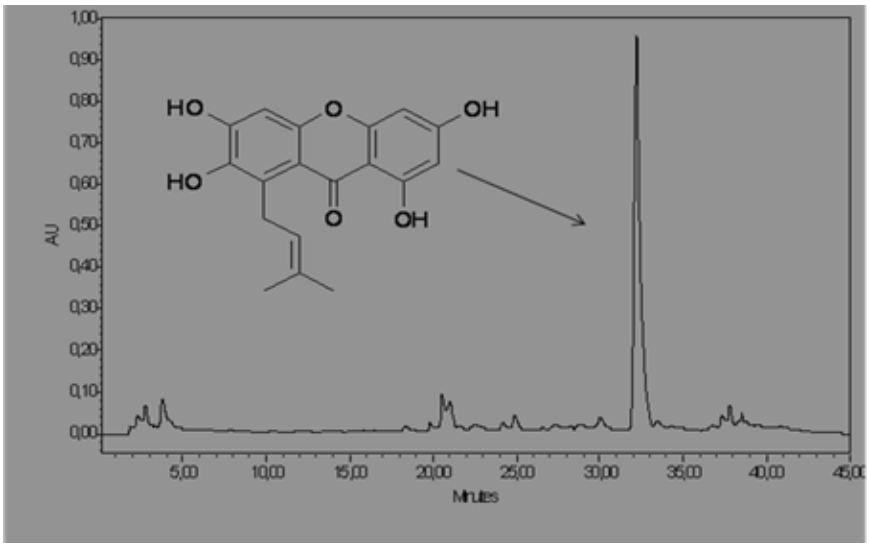
Cell cultures of *H. calycinum* grown in LS medium accumulated a new compound inside the cells upon treatment with yeast extract. The medium contained only traces of this compound. Cell-free extract prepared from 12-hour-elicited cells was analyzed by HPLC (3.11), which confirmed the formation of the new constituent inside the cells after elicitation with yeast extract (Fig. 4.9). Control cells treated only with an equivalent volume of water failed to form the new constituent.

The inducible compound was purified on preparative silica gel plates (3.12) and analyzed by LC-MS. The mass spectrum (Fig. 4.11) and the UV spectrum (Fig. 4.12) of the new compound agreed with those reported for 1,3,6,7-tetrahydroxy-8-prenylxanthone (Fig. 4.10) in the literature (Abd El-Mawla, 2001). The compound was not detected by GC-MS, which may be due to its relatively high polarity.

This compound was also accumulated in cell cultures of *H. androsaemum* upon elicitation with methyl jasmonate (Abd El-Mawla *et al*., 2001) and in cell cultures of *H. perforatum* upon elicitation with yeast extract (Franklin *et al*., 2009).



A



B

Fig. 4.9. HPLC analysis of cell-free extracts from *Hypericum calycinum* cell cultures
A: Control cells (non-treated)
B: Cells treated with yeast extract (3 g/l)

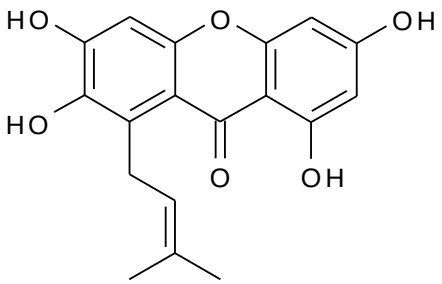


Fig. 4.10. 1,3,6,7-Tetrahydroxy-8-prenylxanthone

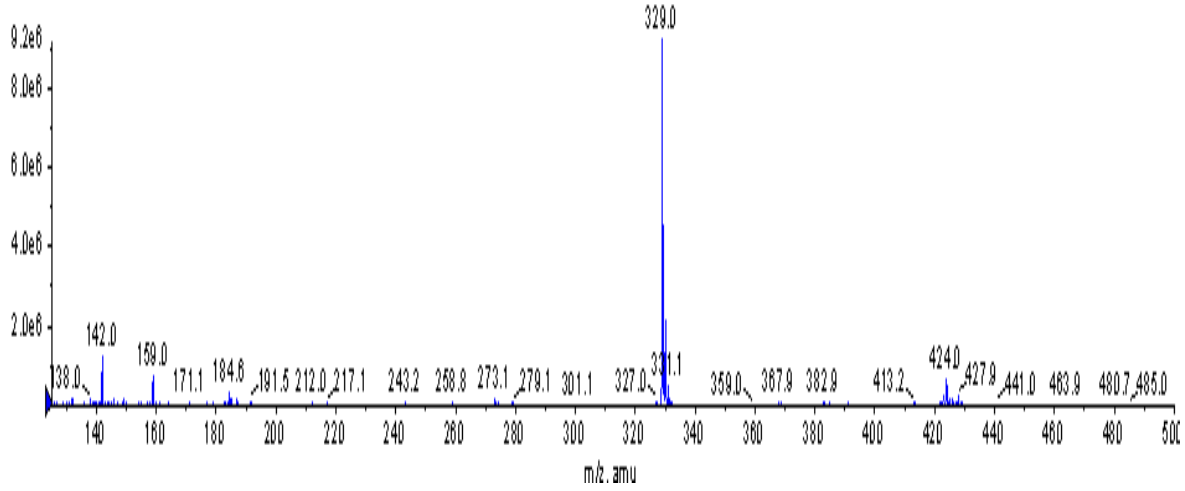


Fig. 4.11. ESI-MS spectrum of 1,3,6,7-tetrahydroxy-8-prenylxanthone from yeast-extract-treated *Hypericum calycinum cell cultures*

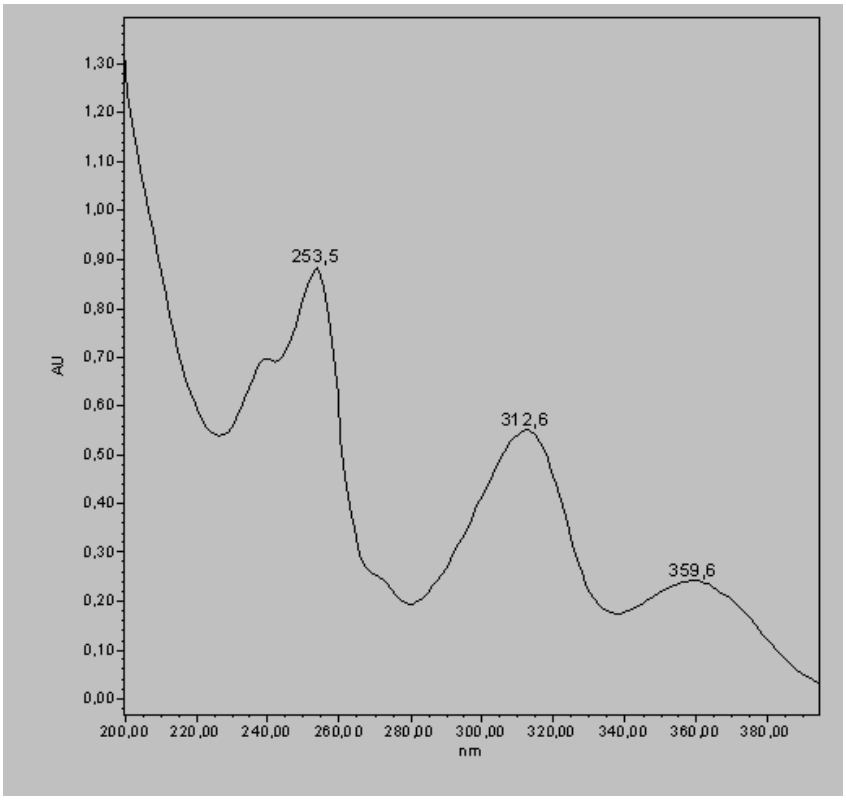


Fig. 4.12. UV/Vis spectrum of 1,3,6,7-tetrahydroxy-8-prenylxanthone

4.2.2.1. Time course changes of 1,3,6,7-tetrahydroxy-8-prenylxanthone accumulation in cell cultures after yeast extract elicitation

The accumulation of the induced compound, 1,3,6,7-tetrahydroxy-8-prenylxanthone, started around 9 h after the onset of elicitation, reached the maximum after 24 hours and decreased afterwards (Fig. 4.13). This might be due to instability and degradation of the product.

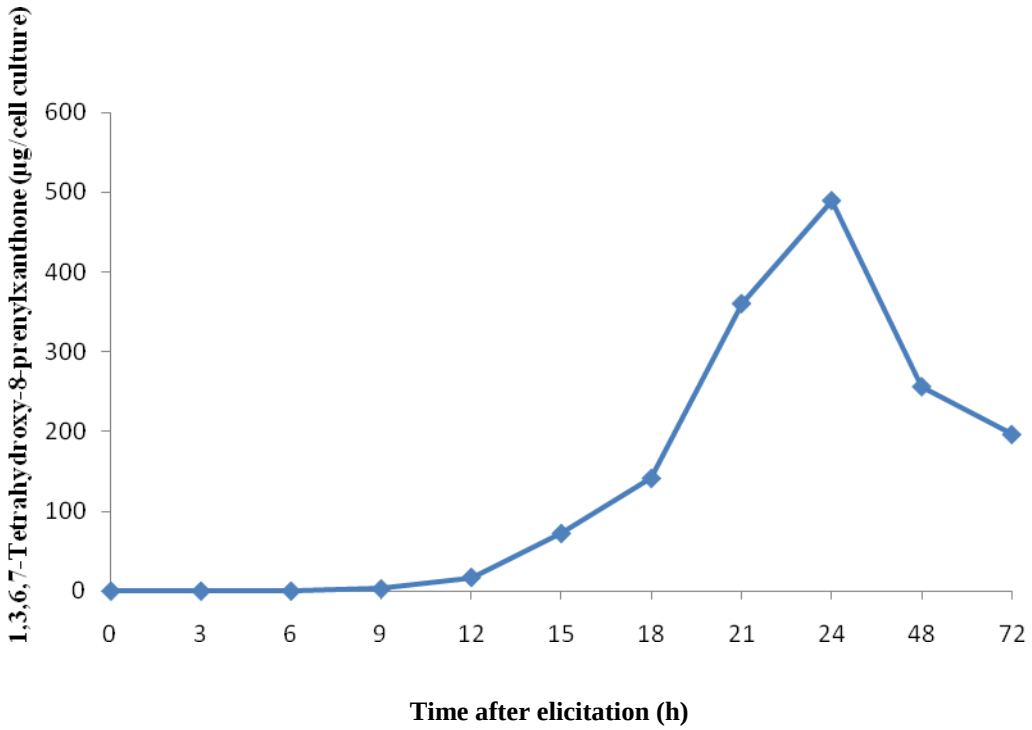


Fig. 4.13. Accumulation of 1,3,6,7-tetrahydroxy-8-prenylxanthone in cell cultures of *Hypericum calycinum* after elicitation with yeast extract. Data are mean values of two independent experiments

4.2.3. Detection of the activity of benzophenone synthase (BPS) in cell cultures of *Hypericum calycinum*

Four and five-day-old cell cultures of *H. calycinum* were treated with yeast extract (3 g/l) and thereafter harvested at three-hour-intervals. The harvested cells were used to extract the proteins (3.16). The protein concentration was measured by Bradford (3.18). The extracts were used to detect BPS activity at 35 °C in 20-min-incubations (3.42). Formation of the enzymatic product, phlorbenzophenone, was analyzed by HPLC. Unexpectedly and repeatedly, no BPS activity was detectable although the cell cultures of *H. calycinum* formed xanthone upon elicitation with yeast extract. BPS appears to be denatured during the extraction procedure.

4.2.4. Cloning of a BPS cDNA from *Hypericum calycinum* cell cultures

4.2.4.1. Extraction of mRNA and reverse transcription

Four-day-old cells were challenged with yeast extract (3 g/l). mRNA was isolated after 6 hours from 100 mg of cells (3.20). One µg mRNA was used for reverse transcription to get cDNA (3.24), which was employed as template to amplify the BPS sequence.

4.2.4.2. Amplification of the BPS cDNA

To amplify a cDNA encoding BPS from *H. calycinum*, *Taq* DNA polymerase and two primers (BPS *H.and* fwd and BPS *H.and* rev) (2.12) derived from the non-translated regions of a BPS cDNA from *H. androsaemum*, a closely related species, were applied. The amplified BPS cDNA was run in an agarose gel (3.8) and had a size of 1200 bp, as it was expected (Fig. 4.14).

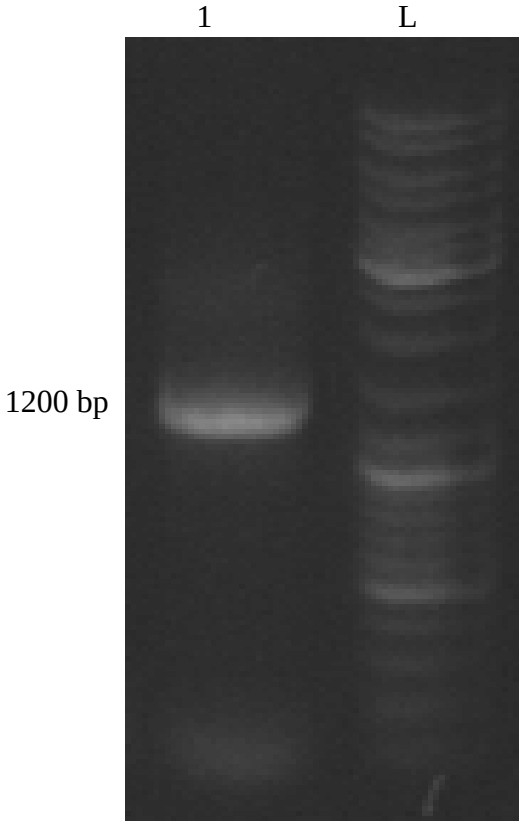


Fig. 4.14. Agarose gel electrophoresis of BPS cDNA from *Hypericum calycinum* cell cultures
1: BPS ORF, L: DNA ladder mix (Fermentas)

4.2.4.3. Gel purification, ligation into T7 vector and sequencing

The BPS cDNA was purified from the agarose gel by using a purification kit (3.9). It was cloned into T7 vector as described in (3.25) and transferred into *E. coli* DH5α (3.29). After white-blue selection, a colony containing the plasmid was grown in 5 ml liquid LB medium (2.8.3) containing 100 µg/ml ampicillin at 37 °C over night. The plasmid was isolated (3.31) and digested with *Eco*RI (3.32) to give two fragments, one of them was the BPS cDNA band at 1200 bp size.

The plasmid without restriction was sent to sequencing (MWG, Germany); in addition one copy was sequenced in our own laboratory. The results were compared with data bank entries (www.ncbi.nlm.nih.gov/BLAST/), which showed that the *H. calycinum* BPS has a high degree of similarity to the *H. androsaemum* BPS. The identity at the amino acid level was 97.7% (Fig. 4.15).

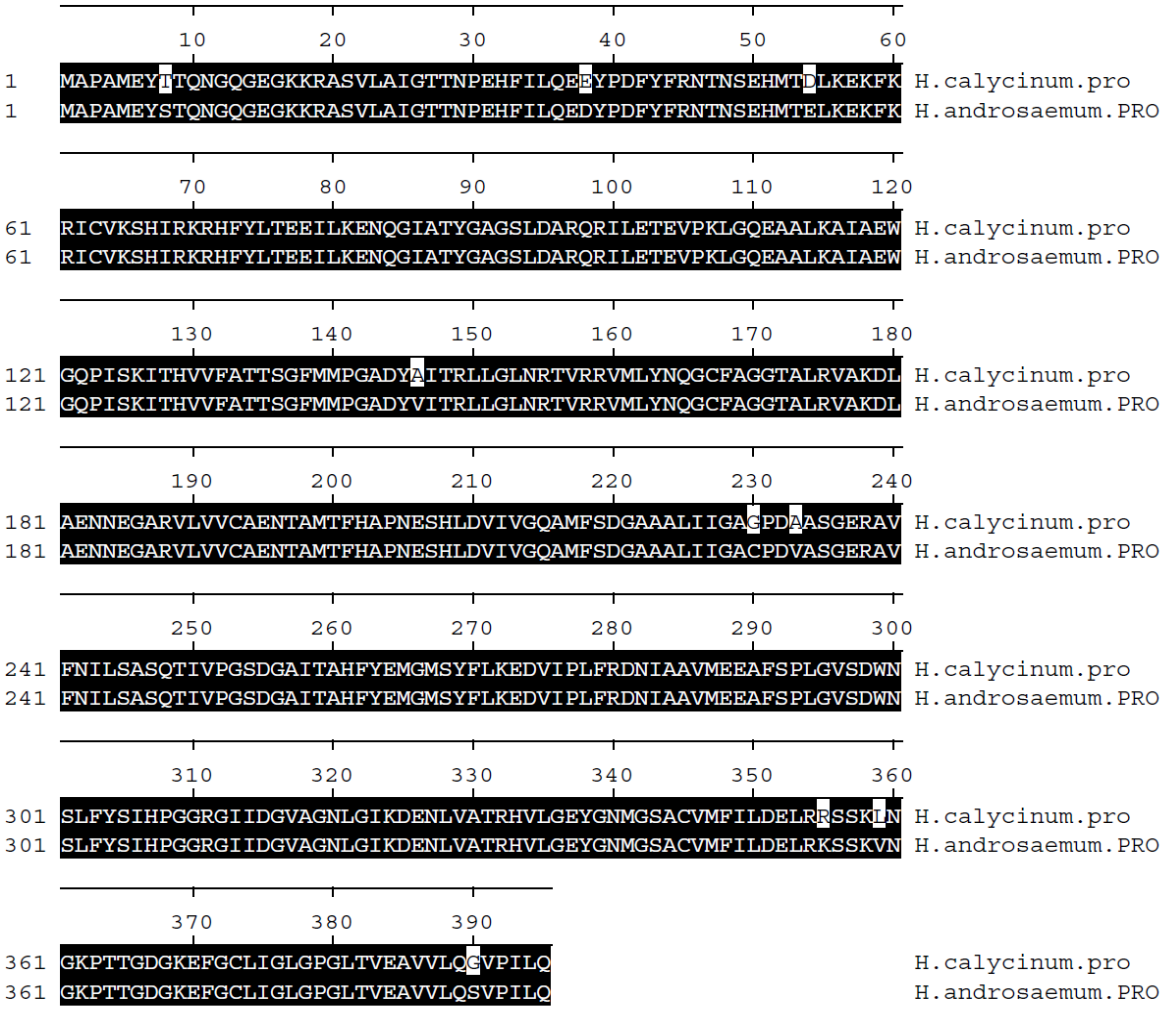


Fig. 4.15. Alignment of BPSs from *Hypericum androsaemum* and *Hypericum calycinum*

4.2.5. Construction of expression plasmid

4.2.5.1. Construction of BPS/pRSET B plasmid

Proofreading *Pfu* DNA polymerase was used to reamplify the BPS ORF from reverse-transcribed mRNA from elicitor-treated cells in a standard PCR assay at an annealing temperature of 60 °C (3.7). The forward primer, BPS Exp fwd, contained a *Nhe*I restriction site and integrated the start codon ATG, whereas the reverse primer, BPS Exp rev, contained a *Kpn*I restriction site directly behind the stop codon (2.12). The restriction sites were introduced to allow the cloning of the BPS ORF into the expression vector pRSET B (Fig. 4.16). The constructed plasmid was sequenced to ensure the presence of the right insert, i.e. the coding region of the BPS cDNA. No frame shifts were detected and the BPS sequence was identical to that obtained by *Taq* DNA polymerase (4.2.4.2).

4.2.5.2. Gel purification, ligation into pRSET B vector and expression

The DNA was purified from the agarose gel using a purification kit (3.9), cloned into pRSET B vector as described in (3.25) and transferred into *E. coli* DH5α (3.29). After selection, a colony including the plasmid was grown in 5 ml liquid LB medium at 37 °C over night. The plasmid was isolated (3.31) and digested with *Kpn*I and *Nhe*I (3.32) to give two fragments, one of which was 1200 bp (Fig. 4.17).

The plasmid which had the right insert was transferred into *E. coli* BL21 (3.36). The bacteria were cultured in LB medium. IPTG was added to induce the expression of the recombinant protein (3.37). The protein concentration was measured by Bradford (3.18).

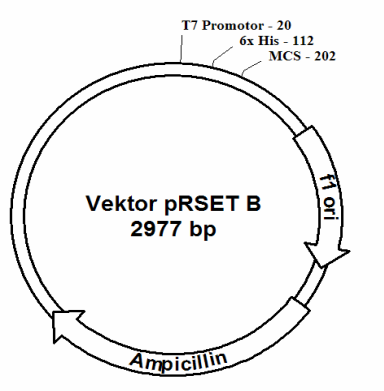


Fig. 4.16. pRSET B vector

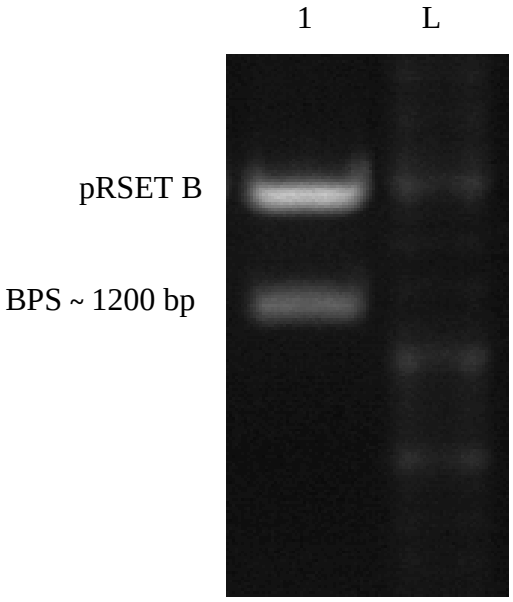


Fig. 4.17. Restriction of BPS/pRSET B plasmid using *Nhe*I and *Kpn*I
1: Digested BPS/pRSET B plasmid, L: DNA ladder mix (Fermentas)

4.2.6. Protein expression

BPS was expressed as His_6-tagged protein with a molecular mass of about 43 kDa and purified by using the Ni-NTA system (3.38 and 3.39). The purification efficiency was examined by SDS-PAGE (3.41) (Fig. 4.18). A single protein band was detected which corresponds to the expected subunit size.

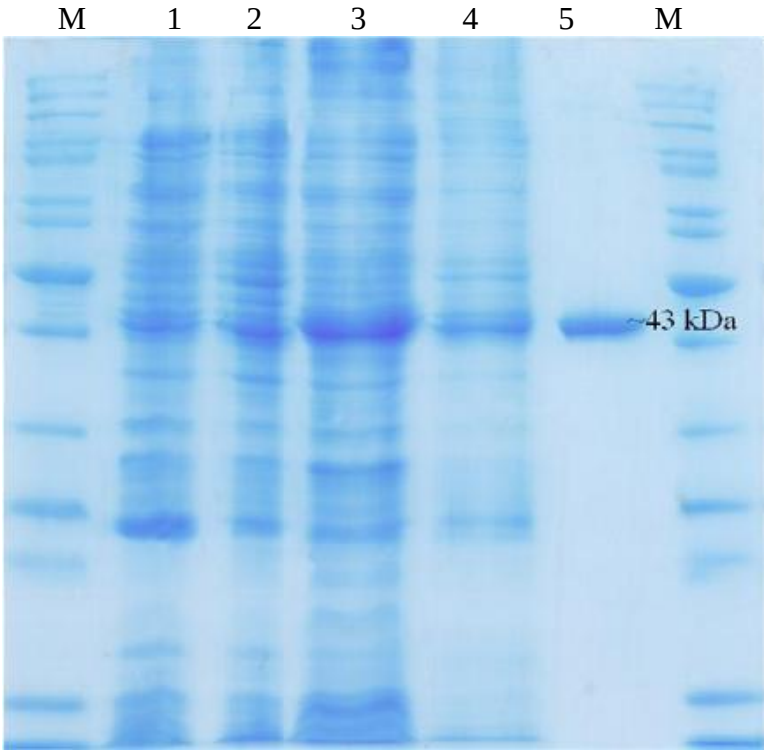
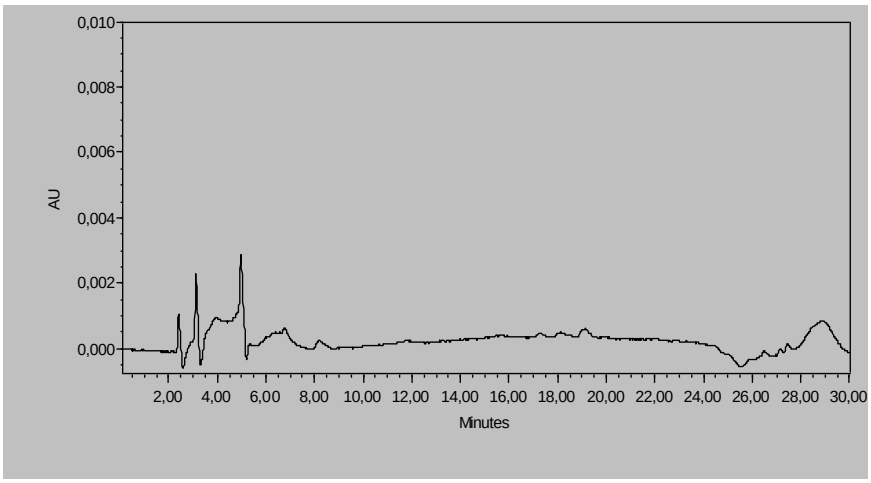


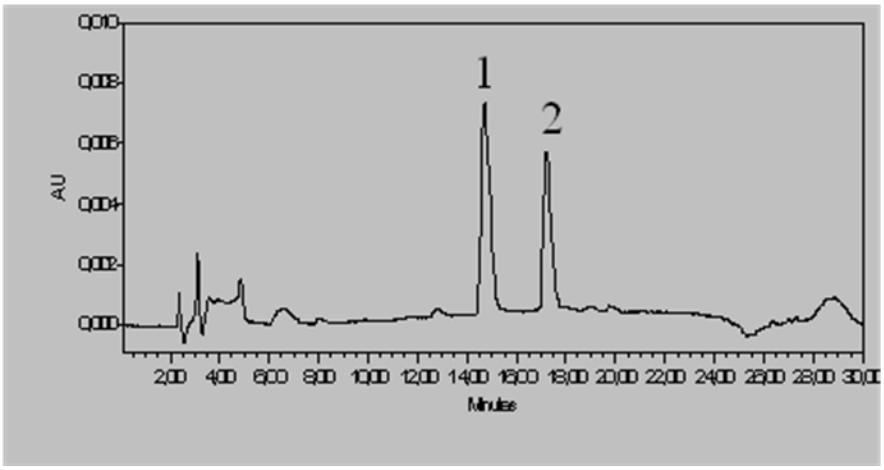
Fig. 4.18. Purification of His_6-BPS using the Ni-NTA system. M: protein marker, 1: pre-induction, 2: post-induction, 3: pellet, 4: supernatant, 5: affinity-purified protein

4.2.7. Detection of benzophenone synthase activity

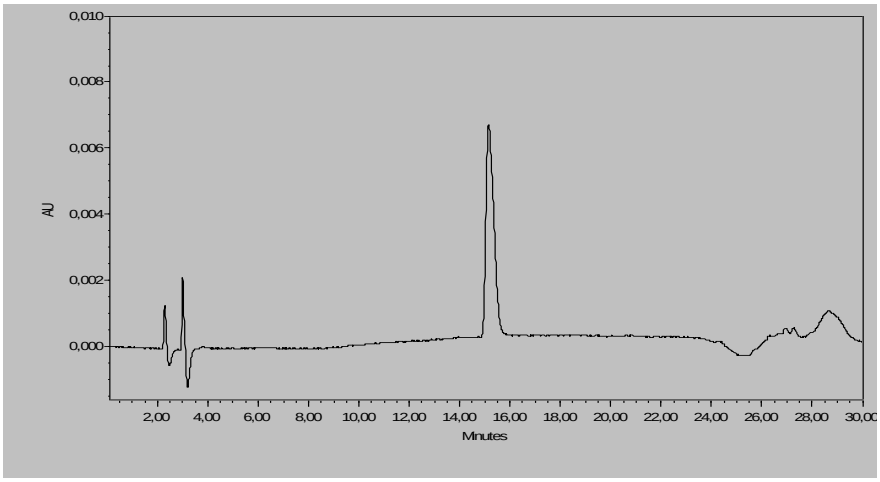
The purified recombinant BPS (3.38) was incubated with benzoyl-CoA and malonyl-CoA for 10 minutes at 35 °C (3.42). BPS activity was confirmed by HPLC analysis (3.14). The HPLC chromatogram showed two enzymatic products: 2,4,6-trihydroxybenzophenone as main product and 6-phenyl-4-hydroxy-2-pyrone as side product (Fig. 4.19). Incubation with heat-denatured protein failed to show any activity in the HPLC chromatogram, indicating the enzymatic origin of the two products. Identification of the enzymatic products was done in comparison to authentic reference compounds by co-chromatography and UV spectroscopy.



A



B



C

Fig. 4.19. HPLC analysis of the activity of the recombinant BPS from *Hypericum calycinum* cell cultures using benzoyl-CoA and malonyl-CoA as substrates
A: Control (boiled protein)
B: Standard assay; main product, 2,4,6-trihydroxybenzophenone (1), side product, 6-phenyl-4-hydroxy-2-pyrone (2)
C: Authentic reference, 2,4,6-trihydroxybenzophenone

4.2.8. Characterization of recombinant BPS from *Hypericum calycinum* cell cultures

4.2.8.1. Determination of temperature, pH and DTT optima

Enzyme activity increases with increasing temperature up to a maximum. At higher temperatures the protein will undergo excessive denaturation, i.e. loss of conformational structure, by affecting intramolecular bonds that stabilize the protein structure. BPS was incubated at 15, 20, 25, 30, 35, 40 and 45 °C for 10 minutes. The product amounts formed were analyzed by HPLC. The optimum incubation temperature for BPS was around 35 °C (Fig. 4.20).

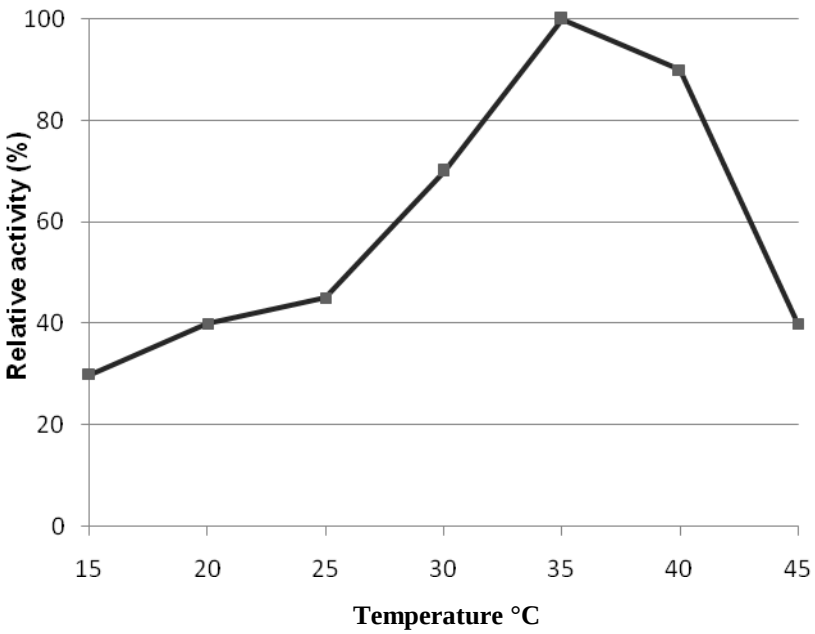


Fig. 4.20. Temperature optimum of BPS. Mean values from two experiments

In addition to temperature, the pH has a massive effect on the enzyme activity. Acids and bases affect the charge of amino acids and may change the functional conformation of the proteins. At 35 °C BPS was incubated at pH 5.5, 6, 6.5, 7, 7.5, 8 and 8.5 for 10 minutes. The product amounts formed were analyzed by HPLC. The pH optimum of BPS was at 7 (Fig. 4.21).

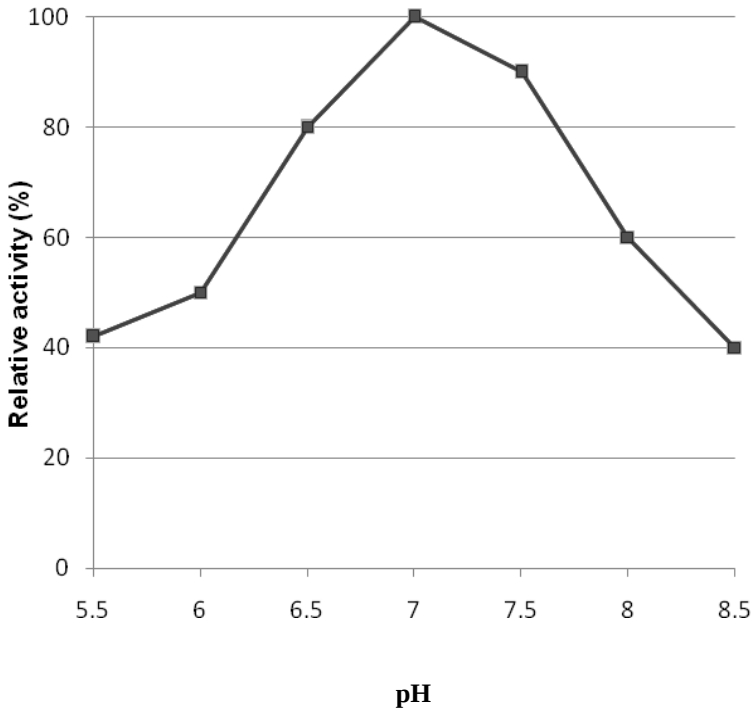


Fig. 4.21. pH optimum of BPS. Mean values from two experiments

Dithiothreitol (DTT) is a reducing agent which stabilizes the SH-groups of cysteine residues at the active site. If disulfide bonds are formed, they can be reduced by DTT. At pH 7, incubations were performed for 10 minutes at 35 °C in the presence of 0, 10, 20, 50 and 100 µM DTT. BPS activity was stimulated by DTT to give maximum formation of 2,4,6-trihydroxybenzophenone at 50 µM. However, side product formation also increased in the presence of DTT (Table 4.2).

Table 4.2. Effect of DTT on *Hypericum calycinum* BPS. Mean values from two experiments

DTT concentration (µM)	Benzophenone formation (% of max.)	Side-product formation (% of max.)
0	61	32
10	72	44
20	84	58
50	100	67
100	92	100

4.2.8.2. Substrate specificity

Enzyme assays were done to test the ability of BPS to use different starter substrates. The products formed were analyzed by HPLC. The most efficient substrate for *H. calycinum* BPS was benzoyl-CoA (Table 4.3). No activity was noticed with *o*-hydroxybenzoyl-CoA, *p*-coumaroyl-CoA and acetyl-CoA. However, other substrates such as *m*-hydroxybenzoyl-CoA and longer aliphatic starters were converted at lower reaction rates.

Table 4.3. Substrate specificity of BPS from *Hypericum calycinum* cell cultures

Substrate	Relative activity (%)
Benzoyl-CoA	100
o-Hydroxybenzoyl-CoA	0
m-Hydroxybenzoyl-CoA	37
p-Coumaroyl-CoA	0
Acetyl-CoA	0
Butyryl-CoA	28
Isobutyryl-CoA	22
Isovaleryl-CoA	12
Hexanoyl-CoA	32
Octanoyl-CoA	7

4.2.8.3. Determination of kinetic data

The kinetic properties of BPS were determined under the optimum conditions, i.e. pH 7 and 35 °C. DTT was omitted due to stimulation of side product formation. The K_m, K_{cat} and K_{cat}/K_m values were calculated for the preferred starter substrate, benzoyl-CoA (Table 4.3), and the extender substrate, malonyl-CoA. Increasing concentrations of benzoyl-CoA were used while keeping the concentration of malonyl-CoA constant at saturation and vice versa.

Benzoyl-CoA conversion by BPS obeyed Michaelis-Menten kinetics (Fig. 4.22). The K_m value for benzoyl-CoA was 10.8 µM (Lineweaver-Burk) (Fig. 4.23), 11.2 µM (Hanes) and 9.5 µM (Eadie-Hofstee). The K_{cat} value was 0.43 min^{-1} and the K_{cat}/K_m value was 0.66 mM^{-1} sec^{-1}.

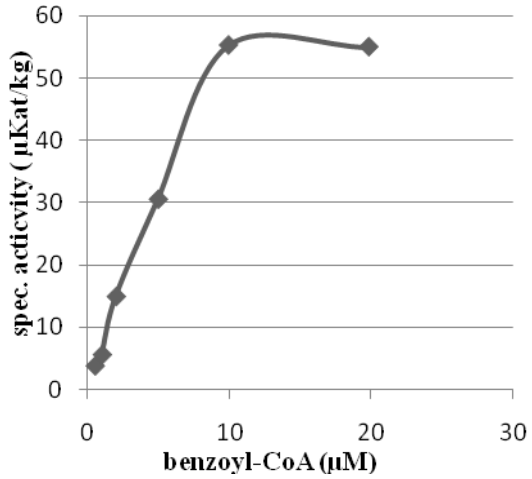


Fig. 4.22. Michaelis-Menten kinetics for benzoyl-CoA conversion

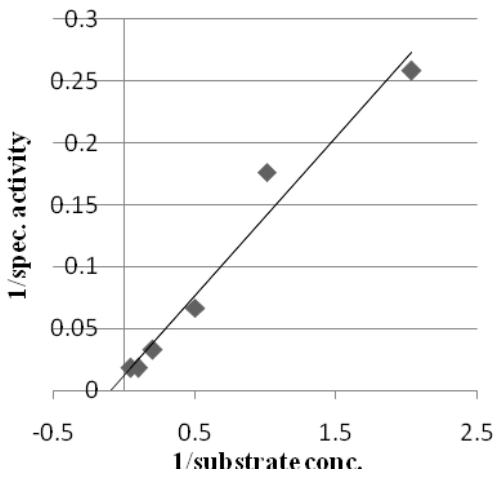


Fig. 4.23. Determination of the K_m value for benzoyl-CoA from a Lineweaver-Burk plot

Conversion of malonyl-CoA also obeyed Michaelis-Menten kinetics (Fig. 4.24). The K_m value for malonyl-CoA was 15.4 µM (Lineweaver-Burk) (Fig. 4.25), 13.9 µM (Hanes) and 15.3 µM (Eadie-Hofstee). The K_{cat} value was 1.11 min^{-1} and the K_{cat}/K_m value was 1.2 $mM^{-1}sec^{-1}$.

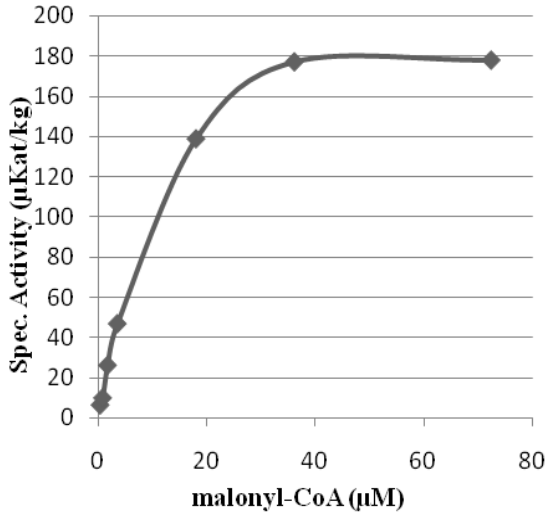


Fig. 4.24. Michaelis-Menten kinetics for malonyl-CoA conversion

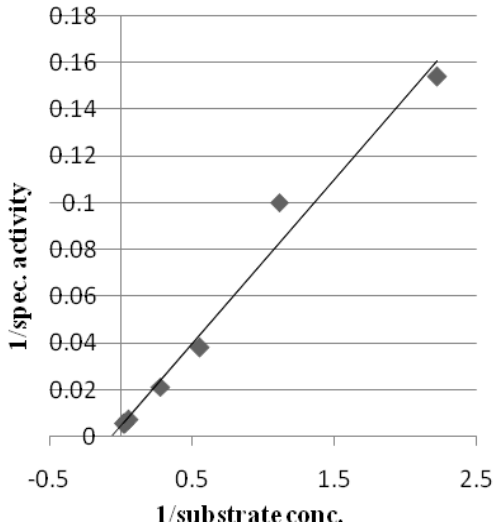


Fig. 4.25. Determination of the K_m value for malonyl-CoA from a Lineweaver-Burk plot

The K_m, K_{cat} and K_{cat}/K_m values for benzoyl-CoA and malonyl-CoA are summarized in Table 4.4.

Table 4.4. Steady-state kinetic parameters for BPS from *Hypericum calycinum* cell cultures

Substrate	k_m [µM]	k_{cat} [min^{-1}]	k_{cat}/k_m [$mM^{-1}sec^{-1}$]
Benzoyl-CoA	10.83	0.43	0.66
Malonyl-CoA	15.38	1.11	1.2

4.2.9. RNA gel blot analysis

Total RNA was isolated from *H. calycinum* cell cultures in three-hour-intervals after yeast extract treatment (3.21), separated by RNA agarose gel electrophoresis (3.22), blotted to a positively charged nylon membrane and hybridized with a 300 bp *BPS*-specific probe (3.23). BPS transcripts started to accumulate 3 h post-elicitation with maximum mRNA levels at 12 h (Fig. 4.26). Thereafter, the transcription rate of the *BPS* gene decreased gradually. As a positive control, the 300 bp fragment of the BPS cDNA was included in the blot analysis. In addition, hybridization was carried out using a 300 bp *CHS*-specific probe, which, however, did not detect any transcripts, indicating that *CHS* expression is not induced upon treatment with yeast extract. No cross-hybridization between the two PKS probes was observed.

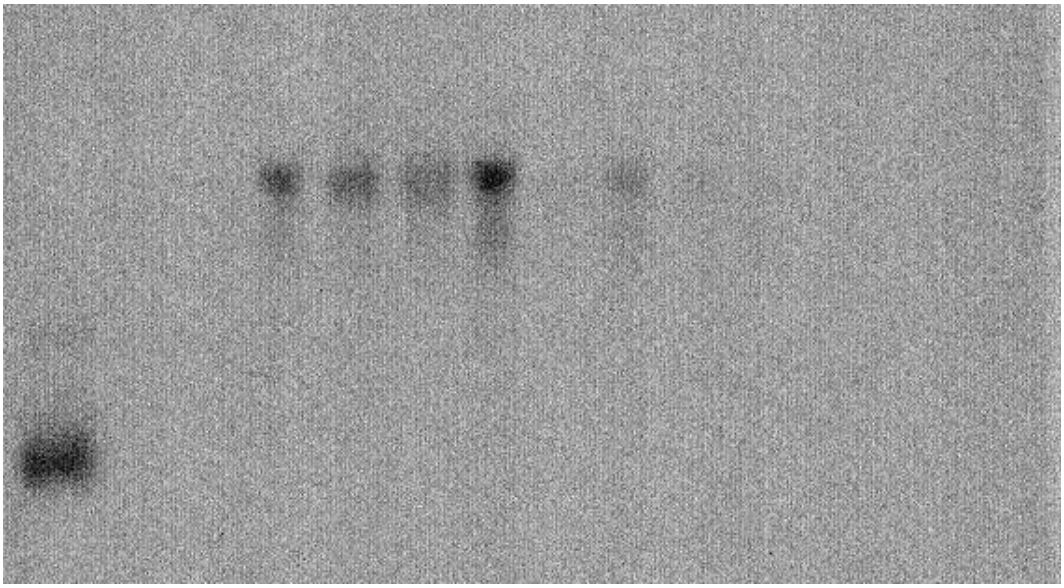


Fig. 4.26. RNA gel blot analysis of *BPS* expression in *Hypericum calycinum* cell cultures after treatment with yeast extract. The Northern blot was hybridized with a *BPS*-specific probe. BPS, 300 bp BPS cDNA fragment, CHS, 300 bp CHS cDNA fragment

5. Discussion

5.1. Transformation of *Hypericum perforatum*

The transformation is an important technique in the field of genetic engineering. It is the insertion of new genetic information into the cell genome to get a genetic alteration in the target cell using an appropriate mechanism. This technique can produce plants that carry new beneficial genetic information. The best-known methods used in many laboratories to produce transgenic plants are *Agrobacterium*-mediated transformation, particle bombardment-mediated transformation and transformation of protoplasts by either electroporation or polyethylene glycol (PEG).

An established protocol for the transformation of *H. perforatum* would be an excellent tool to manipulate the medicinal properties of this plant, such as antidepressant and anticancer activities (Singer *et al*., 1999), and to examine the biosynthetic pathways of different secondary metabolites (Franklin *et al*., 2007), especially hyperforin, which is the most important active constituent in *H. perforatum*. Hyperforin was reported to have antidepressant activity by inhibition of the reuptake of neurotransmitters, such as serotonin and dopamine (Moore *et al*., 2000). It was also postulated that other constituents, such as hypericin and flavonoids, are involved in the antidepressant effect of St. John's wort preparations (Müller, 2003; Butterweck, 2003).

In addition, hyperforin exhibits strong antibacterial activity (Gurevich *et al*., 1971; Schempp *et al*., 1999). The following table shows the minimum inhibitory concentrations (MIC) of hyperforin on bacteria and fungi (Table 5.1). The effect on gram-positive bacteria already occurs at low hyperforin concentrations. However, the compound did not stop the growth of fungi and gram-negative bacteria (Reichling *et al*., 2001).

Table 5.1. Antimicrobial activity of hyperforin against bacteria and fungi (from Reichling *et al*., 2001). MIC, minimum inhibitory concentration

Organism	MIC (µg/ml)
Gram-positive bacteria	
Staphylococcus aureus (multi-resistant)	0.1
Sarcina lutea	0.1
Bacillus subtilis	0.2
Bacillus mycoides	0.2
Mycobacterium phlei	1
Corynebacterium michiganes	1
Streptococcus faecalis	1
Gram-negative bacteria	
Escherichia coli	400
Proteus vulgaris	400
Fungi	
Penicillium chrysogenum	400
Fusarium avenaceum	400
Mucor plumbeum	400
Candida albicans	400

In this work, the effect of *H. perforatum* extracts on the growth of the gram-negative *Agrobacterium* during co-cultivation was clear. However, it is still open if hyperforin, which accumulates in differentiated tissues, is responsible for the loss of virulence of *Agrobacterium*. A previous study proved the antibacterial activity of undifferentiated cell cultures of *H. perforatum*, which should lack hyperforin, during co-cultivation with *Agrobacterium* (Franklin *et al*., 2008 and 2009). The same was true for the present investigation where no successful *Agrobacterium*-mediated transformation was achieved with roots which do not contain hyperforin. Nor were aerial parts transformed.

5.1.1. *Agrobacterium*-mediated transformation

Transformation *via Agrobacterium* is an important tool in modern plant genetic engineering. *Agrobacterium* is the favorite vehicle for gene transfer due to its simplicity, cost-effectiveness and stable integration of foreign genes into the host plant genome (Franklin *et al*., 2007).

A. tumefaciens and *A. rhizogenes*, when interacting with wounded plant cells, induce diseases known as crown gall and hairy roots, respectively, by transferring a small DNA segment (T-DNA) of the tumor-inducing plasmid (Ti) and the hairy root-inducing plasmid (Ri), respectively, into the infected cell, where it is then stably integrated into the host cell genome (la Riva *et al*., 1998).

The Ti/Ri plasmid contains two parts needed for genetic transformation: vir region and T-DNA. The vir region encodes most of the bacterial proteins necessary for the transformation. The bacterial T-DNA is bordered by 25 bp at each end that are important for the transformation system and contains genes that code for opines and phytohormones such as auxin and cytokinin responsible of the uncontrollable growth of the plant cells. Opines are amino acid derivatives used by the *Agrobacterium* as source of nitrogen and carbon (Tzfira *et al*., 2004). The T-DNA transfer initiates at the left border and terminates at the right border.

In the laboratory work, the T-DNA and the vir genes are placed on two small separate plasmids. This system is called a binary vector system. Any gene can be inserted between the two borders of the T-DNA.

Certain requirements have to be present in a reproducible *Agrobacterium* gene transfer system for the production of transgenic plants: (1) the regeneration availability of the infected plant cells; (2) an appropriate condition for T-DNA transferring into these regenerable cells; (3) a selective agent to permit only the regeneration of the transformed plants and a suitable antibiotic (mostly cefotaxime) for killing the *Agrobacterium*; (4) the activation of vir gene expression by phenolic compounds such as acetosyringone; (5) a reporter gene system for detection of successful transfer of the foreign gene into the resulting transgenic plant (Birch, 1997; Wei *et al*., 2000).

In this study, *A. tumefaciens* and *A. rhizogenes* were used for the delivery of foreign DNA into *H. perforatum*. Two strains of *A. tumefaciens* were employed, C58C1/pBIN19/BPS, resistant to kanamycin, rifampicin and gentamycin and AGL1 resistant to bialaphos. The AGL1 strain carries the *GUS* gene for histochemical detection. The *A. rhizogenes* strain LBA1334 resistant to rifampicin was also used.

Acetosyringone was added to the co-cultivation medium of *Agrobacterium* before infection of the explants, which is necessary to obtain transgenic plants. Acetosyringone is a phenolic secondary metabolite secreted by the plant tissues due to injury or stress. *Agrobacterium* recognizes this low molecular weight-signal molecule, which induces expression of the vir genes, leading to the activation of the T-DNA transfer to the plant cells (Zambryski, 1988).

Thus, plant transformation starts with the induction of the expression of the *Agrobacterium* vir region by acetosyringone, secreted from the wounded parts (Citovsky *et al*., 1992). VirA detects these compounds and phosphorylates VirG, which leads to activation of further vir gene transcription (Zupan and Zambryski, 1995). The induced VirD1 and VirD2 proteins recognize the border sequences and cut out the lower DNA strand producing a single-stranded T-DNA. The VirD2 protein binds covalently to the 5´ end of the T-DNA to lead it to the hosted cell. This T-DNA is covered by many molecules of the VirE2 protein forming the T-complex and preventing T-DNA degradation (Zupan and Zambryski, 1995). The T-DNA is then exported to the host cell through a channel that is formed by the VirB and VirD4 proteins (Tzfira *et al*., 2004). The foreign DNA is recognized in the plant, completed as a double strand and integrated into the genome using the plant repair mechanisms (Li *et al.*, 2005).

A. tumefaciens was successfully used to transfer different genes to a wide range of plant species. However, there are several reports of low transformation ability in some other species (Franklin *et al*., 2007). So far, no report has shown the ability of *A. tumefaciens* to infect species of the genus *Hypericum* including the most important member *H. perforatum*, which is an important medicinal plant worldwide.

Many trials were made in this study including changes of the transformation conditions to overcome *H. perforatum* recalcitrance to *Agrobacterium.* This resistance was produced either by the elimination of the *Agrobacterium* or by necrosis in the explants after infection. One reason might be the antibacterial activity of hyperforin and hypericin that are located in

the aerial parts of *H. perforatum*, such as flowers and leaves, and cause a decrease of the *Agrobacterium* viability during co-cultivation (Franklin *et al*., 2007).

Another reason might be the formation of inducible secondary metabolites as a plant defense response against pathogens. Salicylic acid is a signal molecule regulating the plant defense system in response to a wide variety of pathogens. It can shut down the expression of *Agrobacterium* vir genes and directly affect the infection process (Yuan *et al*., 2007). Xanthones appear to serve as phytoalexins in various species. They exhibit antimicrobial and antioxidant activities (Beerhues *et al*., 2009). They might also be a reason of the *Agrobacterium* mortality during co-cultivation. In this study 1,3,6,7-tetrahydroxy-8-prenylxanthone was found to accumulate rapidly in cell cultures of *H. calycinum* upon treatment with yeast extract. A similar induction of xanthone formation was previously observed in cell cultures of two *Centaurium* species (Beerhues and Berger, 1995). In *H. perforatum* cell cultures, xanthone production and antimicrobial efficiency were significantly increased after elicitation with *A. tumefaciens*, causing reduction in the *Agrobacterium* viability during co-cultivation (Franklin *et al*., 2009).

On the other hand, two strains of *A. rhizogenes*, ATCC 15834 and A4 were able to induce hairy roots in two *Hypericum* species, *H. tomentosum* and *H. tetrapterum*. (Komarovska *et al*., 2009). Furthermore, hairy roots in *H. perforatum* were induced using the two *A. rhizogenes* strains 15834 and 11325 (Di Guardo *et al*., 2003). The successful transformation was proved by PCR amplification of the *rol*C and *vir*C1 genes. Another transformation event of *H. perforatum* using *A. rhizogenes* was achieved using the strain A4M70GUS. After infection of shoots with this strain, hairy roots were induced and the GUS activity was histochemically demonstrated (Vinterhalter *et al*., 2006).

These results raise the question as to differences between *A. tumefaciens* and *A. rhizogenes* in their susceptibility to the *H. perforatum* defense mechanism. It might be that some *A. rhizogenes* strains are less sensitive to the inducible phytoalexins or to the pre-formed constituents of *H. perforatum*, such as hyperforin and hypericin. However, this contradicts a study showing that *H. perforatum* recognizes *A. rhizogenes* as a potential pathogen which rapidly evokes defense responses, leading to dramatic reduction of *Agrobacterium* viability (Franklin *et al*., 2008).

5.1.2. Particle bombardment-mediated transformation

The direct gene transfer *via* particle bombardment allows the transformation of plants that are non-susceptible to *Agrobacterium* and became the second most widely used technique for plant transformation (Jähne *et al*., 1995). This technique is quick but relatively expensive. The integration of the foreign DNA into the plant cell genome is, in contrast to the *Agrobacterium*-mediated transformation, not easily achieved. A number of important species were transformed by particle bombardment including rice (Christou *et al*., 1991) and wheat (Vasil *et al*., 1991).

H. perforatum cell cultures were transformed using particle bombardment and a foreign gene construct containing the *GUS* gene was integrated into the plant cell genome. Transgenic plants were produced. PCR analysis and histochemical GUS assays proved the successful transformation (Franklin *et al*., 2007). However, the efficiency of particle bombardment in leaves of *H. perforatum* was low (Feye, 2007).

One goal of the transformation efforts made with *H. perforatum* is to manipulate the biosynthetic pathways involving polyketide synthases as key enzymes because all active secondary metabolites of the medicinal plant are derivatives of polyketide metabolism.

5.2. Type III polyketide synthases

Plant polyketide synthases (type III PKSs) catalyze the formation of natural secondary metabolites by condensation of multiple acetyl units from malonyl-CoA with a specific starter substrate into polyketides, many of which exhibit interesting pharmacological properties (Schröder, 1999). Formation of polyketides shares similarities to the biosynthetic pathway of fatty acids. Type III PKSs are homodimers of about 84 kDa, which catalyze all steps of the biosynthesis, i.e. decarboxylation, condensation, cyclization and aromatization, at two functionally independent active sites (Austin and Noel, 2003).

In *Hypericum* species four PKSs were detected: chalcone synthase (CHS), isobutyrophenone synthase (BUS), benzophenone synthase (BPS) and octaketide synthase (OKS) (Klingauf *et al*., 2005; Karppinen *et al*., 2008).

Type III PKSs form an amazing number of natural products by varying the starter substrate (aliphatic or aromatic), the number of acetyl additions (1–7) and the mechanism of ring formation used to cyclize linear polyketide intermediates (Claisen condensation, aldol condensation, or heterocyclic lactone formation) (Austin and Noel, 2003; Flores-Sanchez and Verpoorte, 2009). For example, benzophenone synthase (BPS) and biphenyl synthase (BIS) use benzoyl-CoA as starter molecule and form the same tetraketide intermediate after the reaction with three molecules malonyl-CoA as extender (Fig. 5.1). BIS is a type III PKS which was characterized and cloned from elicitor-treated *Sorbus aucuparia* cell cultures (Liu *et al*., 2004 and 2007). While BPS cyclizes the linear intermediate *via* an intramolecular C6→C1 Claisen condensation to form phlorbenzophenone, BIS catalyzes an intramolecular C2→C7 aldol condensation and decarboxylative elimination of the terminal carboxyl group to give 3,5-dihydroxybiphenyl (Beerhues and Liu, 2009).

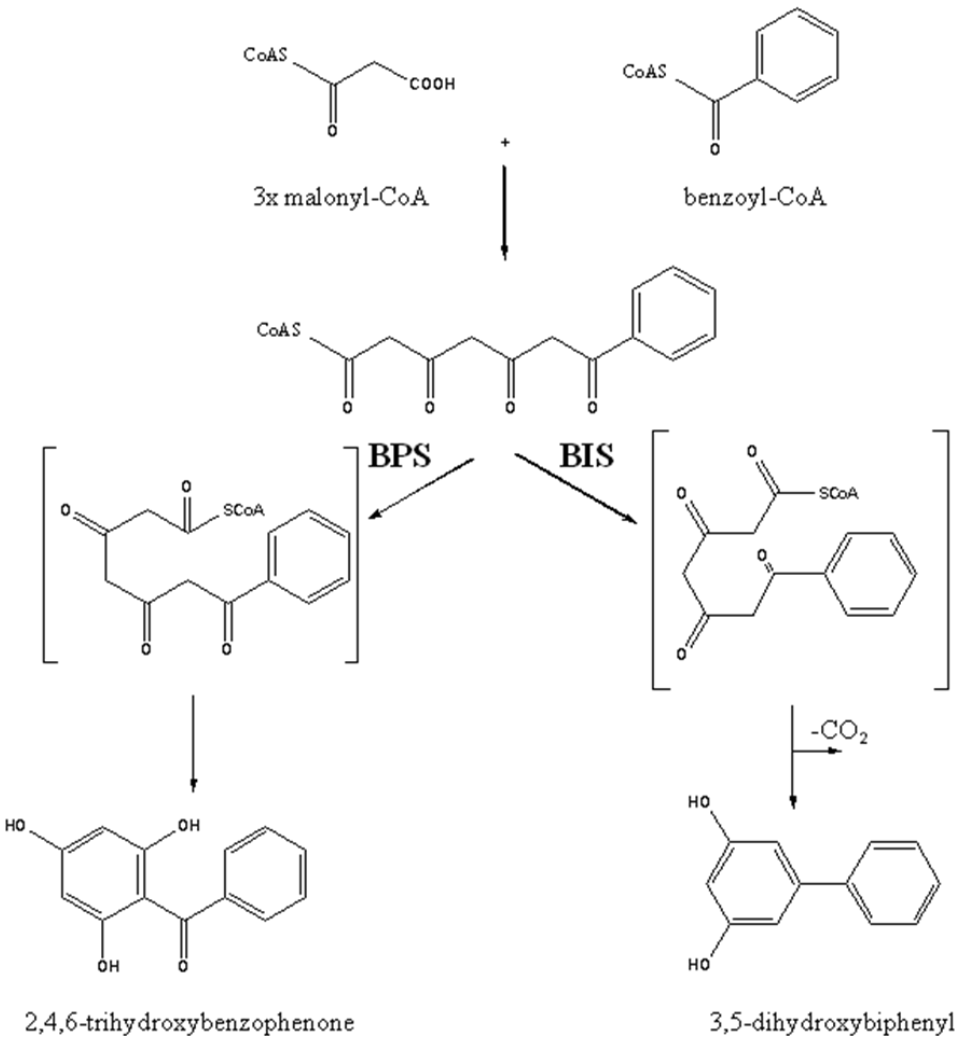


Fig. 5.1. Reactions of benzophenone synthase and biphenyl synthase (modified after Liu *et al*., 2004)

The best-known member of the type III PKS superfamily is chalcone synthase (CHS) which is the first functionally and structurally described PKS (Ferrer *et al*., 1999). CHS forms the precursor of flavonoids which play important functions in plants (Liu *et al*., 2003). CHS uses *p*-coumaroyl-CoA as starter and three molecules of malonyl-CoA as extender to form a tetraketide intermediate (Fig. 5.2), which is cyclized by Claisen condensation to give naringenin chalcone (Liu *et al*., 2003).

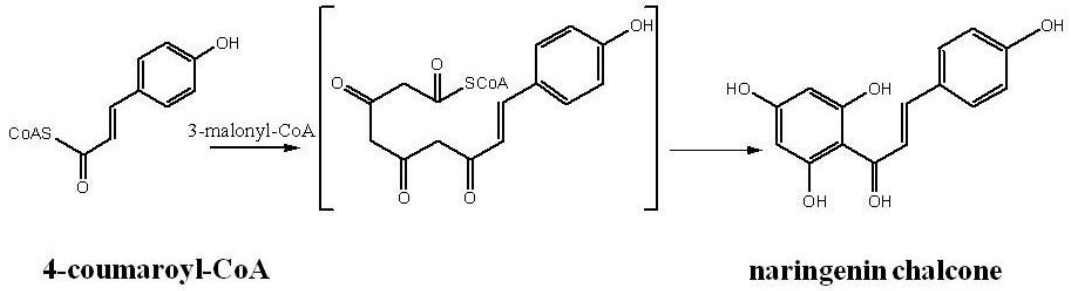


Fig. 5.2. Reaction of chalcone synthase (from Klingauf *et al*., 2005)

Isobutyrophenone synthase (BUS) is responsible for the formation of the hyperforin skeleton (Beerhues, 2006). BUS catalyzes the condensation of isobutyryl-CoA with three molecules of malonyl-CoA to give a tetraketide intermediate which is cyclized by Claisen condensation, forming phlorisobutyrophenone (Klingauf *et al*., 2005). This aromatic intermediate undergoes a series of prenylation reactions, finally yielding hyperforin (Fig. 1.15).

In *H. calycinum* cell cultures, when grown in BDS medium in the dark, an increase in the formation of adhyperforin and, to a lower extent, hyperforin was preceded by an increase in BUS activity (Kligauf *et al*., 2005). The activity of prenyltransferases was also studied in *H. calycinum* cell cultures. After incubation of phlorisobutyrophenone and dimethylallyl diphosphate (DMAPP) with cell-free extracts from three-day-old *H. calycinum* cell cultures, an enzymatic product (dimethylallylphlorisobutyrophenone) was detected by HLPC and GC-MS analysis (Boubakir *et al*., 2005).

The successful transfer of the *BPS* gene from *H. androsaemum* into *H. perforatum* and its systemic expression might lead to formation of phlorbenzophenone, which in turn might be used as a substrate for the prenyltransferases, resulting in the biosynthesis of hyperforin derivatives that possibly exhibit similar pharmacological activity but less drug-drug interaction potential.

5.2.1. Benzophenone synthase

In this work, a BPS cDNA was cloned from *H. calycinum* cell cultures and the recombinant protein was heterologously expressed and characterized. BPS catalyzes a central step in the biosynthesis of xanthones, which exhibit wide pharmacological properties. BPS catalyzes, as mentioned above, the formation of 2,4,6-trihydroxybenzophenone (Beerhues *et al*., 1997) (Fig. 5.3). This metabolite is converted to 2,3',4,6-tetrahydroxybenzophenone by benzophenone 3'-hydroxylase (Schmidt and Beerhues, 1997). Finally, xanthone synthase, a cytochrome P450 enzyme, catalyzes the formation of 1,3,7-trihydroxyxanthone from 2,3',4,6-tetrahydroxybenzophenone (Peters *et al*., 1998). BPS from cell cultures of *Centaurium erythraea* (Gentianaceae) prefers 3-hydroxybenzoyl-CoA as starter substrate, yielding immediately 2,3',4,6- tetrahydroxybenzophenone (Beerhues, 1996), which is not cyclized to 1,3,7-trihydroxyxanthone but to the isomeric 1,3,5-trihydroxyxanthone by the cytochrome P450 enzyme in *C. erythraea* (Schmidt *et al*., 2000). 3-Hydroxybenzoic acid in this species is directly derived from the shikimic acid pathway, whereas benzoic acid in *H. androsaemum* originates from cinnamic acid by C_2 side-chain degradation (Abd El-Mawla *et al*., 2001; Abd El-Mawla and Beerhues, 2002).

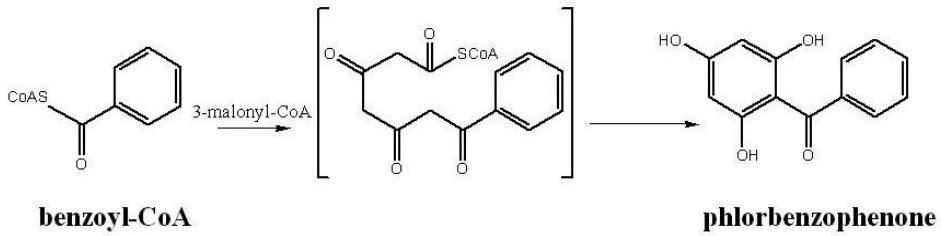


Fig. 5.3. Reaction of benzophenone synthase (from Klingauf *et al*., 2005)

Beside intramolecular cyclization of benzophenones to xanthones, benzophenones can undergo polyprenylation to give derivatives that have interesting pharmacological properties, such as guttiferone F which exhibits antimicrobial and anti-HIV activities (Cuesta Rubio *et al*., 1999; Fuller *et al*., 1999) and sampsonione A, which displays cytotoxic activity (Hu and Sim, 2000). The first BPS cDNA was cloned from *H. androsaemum* cell cultures and heterologously expressed in *E. coli*, followed by characterization of the recombinant protein

(Liu *et al*., 2003). When comparing the sequences of the BPSs from the two *Hypericum* species, only nine amino acids were different. The identity was 97.7%. The homology to other members of the type III PKS family was lower (53-63%).

Similar to *H. androsaemum* BPS, *H. calycinum* BPS has an ORF of 1188 bp that codes for a 395 amino acids protein of 42.8 kDa (Liu *et al*., 2003). The recombinant *H. calycinum* BPS preferred benzoyl-CoA as starter substrate. 3-Hydroxybenzoyl-CoA was used as the second best starter substrate giving 37% relative activity, while BPS from *H. androsaemum* reached 57% relative activity with the same substrate (Klundt *et al*., 2009). The BPSs from both *H. calycinum* and *H. androsaemum* did not accept 4-coumaroyl-CoA as starter, which, however, is the preferred starter for CHS.

The optimal reaction conditions (30 °C, pH 7) were comparable with the published data for the *H. androsaemum* BPS (Liu *et al*., 2003). However some differences were noticed by comparing the kinetic data of the two BPSs. For *H. calycinum* BPS, the K_m for benzoyl-CoA was 10.8 µM and that for malonyl-CoA was 15.38 µM. The K_{cat} value was 0.43 min^{-1} for benzoyl-CoA and 1.11 min^{-1} for malonyl-CoA, resulting in a K_{cat}/K_m value of 0.66 $mM^{-1}sec^{-1}$ for benzoyl-CoA and 1.2 $mM^{-1}sec^{-1}$ for malonyl-CoA.

The functional behavior of *H. androsaemum* BPS was changed by a single amino acid substitution in the active site cavity, which transformed BPS into phenylpyrone synthase (PPS). The point mutation modulated between the formation of a tetraketide product derived from Claisen condensation and a triketide product derived from lactone formation (Klundt *et al*., 2009). The replacement of a threonine at position 135 with a leucine converted BPS into a functional phenylpyrone synthase (Klundt *et al*., 2009). The T135L mutation not only changed the product specificity but also the substrate specificity of BPS. 3-Hydroxybenzoyl-CoA was the second best starter substrate for the wild-type enzyme but a poor starter molecule for the mutant enzyme (Klundt *et al*., 2009).

6. Summary

- Extracts from the medicinal plant *H. perforatum* (St. John's wort; Clusiaceae) are widely used in the treatment of mild to moderate depression. In addition, they exhibit anticancer, anti-inflammatory and antiviral activities. The efficacy was demonstrated in many clinical studies.

- Hyperforin is the major antidepressant constituent. It exhibits a broad inhibitory effect on the neuronal reuptake of serotonin, noradrenaline, dopamine and γ-aminobutyric acid (GABA), resulting in the antidepressant effect. Hyperforin is mainly found in fruits, leaves and flowers.

- Hyperforin is so far defiant to chemical synthesis. Therefore, it was thought to manipulate the biosynthesis of hyperforin by genetic engineering, which might lead to formation of hyperforin-like molecules.

- Many trials under different conditions were done to establish a protocol for the transformation of *H. perforatum* using *A. tumefaciens* or *A. rhizogenes*. However, all these efforts failed, although *Agrobacterium* is able to stably integrate the T-DNA into the genome of numerous plant species. *H. perforatum* may be recalcitrant due to the strong antibacterial activity of its pre-formed constituents or the effectiveness of the induced defense responses against pathogens.

- BPS is the key enzyme of benzophenone and xanthones biosynthesis. A BPS cDNA was cloned from cell cultures of *H. calycinum* treated with yeast extract and heterologously expressed in *E. coli*. The recombinant protein (395 amino acids and 42.8 kDa) was characterized. BPS prefers benzoyl-CoA as a starter substrate, leading to formation of 2,4,6-trihydroxybenzophenone.

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